OPTIMIZATION OF SURVIVIN DIMERIZATION INHIBITORS FOR THE TREATMENT OF DOCETAXEL-RESISTANT PROSTATE CANCER

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DEDICATION

This dissertation is dedicated to Glenda (Mom), Rob (Dad), Samantha (Sister), and Sarah (Fiancé). I am only at this point in my journey because of their unconditional love and support. I will forever be grateful for all that they have done for me.

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Robert Craig Peery

OPTIMIZATION OF SURVIVIN DIMERIZATION INHIBITORS FOR THE TREATMENT OF DOCETAXEL-RESISTANT PROSTATE CANCER

Despite therapeutic advancements, prostate cancer remains the second most common cause of cancer-related mortality in men. Docetaxel is the first cytotoxic agent to show modest improvements in overall survival rate in patients with metastatic prostate cancer. Unfortunately, over half of these patients do not respond to treatment and ultimately all develop resistance. The mechanism mediating docetaxel resistance remains unknown. Survivin has a classical biological role in cancer, in fact survivin has been shown to be overexpressed in almost every solid tumor and is associated with drug resistance and clinically aggressive disease. In these studies I demonstrate that docetaxel resistant cells have overexpression of survivin compared to sensitive parental cells, knockdown of survivin decreases docetaxel resistance, and stable overexpression of survivin increases resistance to docetaxel. The data in these studies suggest that survivin is likely implicated in docetaxel resistance and treatment with a direct survivin inhibitor may sensitize resistant cells to docetaxel. To this end the evaluation and optimization of two different backbones of survivin inhibitors was performed. One such inhibitor identified is LQZ-7-3 which decreases survivin level via proteasome degradation, leads to apoptosis of cells, and showed efficacy in a prostate cancer xenograft model *in vivo* when given in an oral formulation. LQZ-7-3 showed strong specificity to survivin versus other IAP family members at the protein level. Another inhibitor, LQZ-7F-1, demonstrated nanomolar inhibition of

cancer cell growth and similar effects on survivin. Both compounds synergized with docetaxel in vitro warranting future *in vivo* efficacy studies as a combinatorial therapy. Overall, our findings indicate survivin is a significant contributor to docetaxel resistance in metastatic prostate cancer at the molecular level and survivin inhibitors may prove efficacious as a new therapy to sensitize cancer cells to chemotherapies.

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ADT	androgen deprivation therapy
ALL	acute lymphocytic leukemia
AML	acute myeloid leukemia
ANOVA	analysis of variance
AR	androgen receptor
Bcl-2	b-cell lymphoma 2
BIR	baculovirus IAP repeat
BPH	benign prostatic hyperplasia
CD8	cluster of differentiation 8
Cdc2	cell division cycle protein 2
cDNA	complementary deoxyribonucleic acid
cFLIP	cellular FLICE-like inhibitory protein
CHX	cycloheximide
CI	combination index
CPC	chromosomal passenger complex
CREB	cAMP response element binding
DISC	Death Inducing Signaling Complex
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Doc	docetaxel
ECL	enhanced chemiluminescence
ERG	ETS-related gene

FBS	fetal bovine serum
FDA	food and drug administration
FITC	fluorescein isothiocyanate
G418	geneticin
Glu	glutamic acid
H&E	hematoxylin and eosin
HBXIP	Hepatitis B-X-Interacting Protein
HIF1α	hypoxia inducible factor 1 alpha
HIV	human immunodeficiency virus
Hsp	heat shock protein
HTS	high throughput screening
IAP	inhibitor or apoptosis
IC_{50}	half maximal inhibitory concentration
IUSM	Indiana University School of Medicine
LC-MS	liquid chromatography-mass spectrometry
Leu	leucine
Lipo	lipofectamine
M2H	mammalian two hybrid
mCRPC	metastatic Castration Resistant Prostate Cancer
mg	milligram
mRNA	messenger ribonucleic acid
MTD	maximum tolerated dose
NF-κB	nuclear factor kappa B

nm.	nanometer
P-gp	p-glycoprotein
p53	tumor protein 53
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD	pharmacodynamics
Phe	phenylalanine
PIN	prostatic intraepithelial neoplasia
PK	pharmacokinetics
PMSF	phenylmethylsulfonyl fluoride
PSA	prostate specific antigen
PTEN	phosphatase and tensin homolog
Rb	retinoblastoma
RLU	relative light unit
RNA	ribonucleic acid
RPM	revolutions per minute
RPMI	Roswell Park Memorial Institute
SAR	structure activity relationship
SDS	sodium dodecyl sulfate
SEAP	secreted embryonic alkaline phosphatase
siRNA	small interfering ribonucleic acid
SMAC	second mitochondria-derived activator of caspase
Sp1	specific protein 1

STAT3	signal transducer and activator of transcription 3
$t_{1/2}$	half-life
TBS	tris-buffered saline
TGF-β	transforming growth factor beta
TMPRSS2	transmembrane protease, serine 2
Trp	tryptophan
μM	micromolar
v/v	volume by volume
VEGF	vascular endothelial growth factor
w/v	weight by volume
WB	western blot
XIAP	X-linked IAP

CHAPTER 1. INTRODUCTION

1.1 Prostate Cancer

1.1.1 Prostate Biology, Statistics, Cancer Development, Natural History

The prostate is a male-specific, walnut-sized exocrine gland located between the bladder and the penis and just in front of the rectum. Biologically, the prostate is responsible for secreting and supplying a slightly alkaline fluid that make up roughly thirty percent the volume of sperm. This fluid contains a cocktail of proteins, minerals, and enzymes that helps nourish, protect, and escort sperm to fertilize an egg [1]. The prostate surrounds the urethra, a tube that runs from the bladder through the length of the penis which is responsible for transporting semen and urine out of the body [2]. The entire prostate itself is encapsulated by a bundle of collagen, elastin, and large amounts of smooth muscle referred to as the prostatic capsule [3].

The prostate has been historically categorized as having a structure consisting of 4 major areas, with three unique zones that are histologically distinct and anatomically independent (**Figure 1)** [4]. These areas are defined as the non-glandular fibromuscular stroma that surrounds the organ, the peripheral zone, the transition zone, and the central zone [5]. The peripheral zone is the outermost zone of the prostate that is closest to the rectum. It is the largest zone of the prostate and is the zone that is able to be felt during a digital rectal examination. This is the zone of origin for 70-80% of all prostate cancer tumors [6]. The transition zone surrounds the prostatic urethra and comprises about 20% of the gland until the age of 40, in which it begins to swell and impinge on the

urethra in a condition referred to as Benign Prostatic Hyperplasia (BPH). The transition zone is the place of origin for 10-20% of tumors [7]. The final zone of the prostate, the central zone, is a very rare location for tumor development with ~2.5% of cancers arising in this zone. However, these central zone cancers tend to be highly aggressive and more invasive [8]. The androgen receptor (AR) signaling pathway is the critical regulator of prostate development and homeostasis [9].

Histologically, the prostate is tubulo-alveolar gland with two major generic cell types: stromal and epithelial. The stromal compartment of the prostate contains a multitude of different cell types, but the most abundant cells are smooth muscle cells and fibroblasts. The pseudostratified epithelium contains three differentiated epithelial cell types: luminal, basal, and neuroendocrine [10]. The luminal epithelium cells form a continuous layer of polarized columnar cells that produce important secretory factors and proteins and express high levels of androgen receptor [11]. Underneath the luminal layer, is a basal layer of cuboidal epithelial cells that are not secretory and are involved in replenishing the prostate tissue [12]. The separation of the stromal and epithelial compartments is formed by a large basement membrane consisting of extracellular matrix proteins.

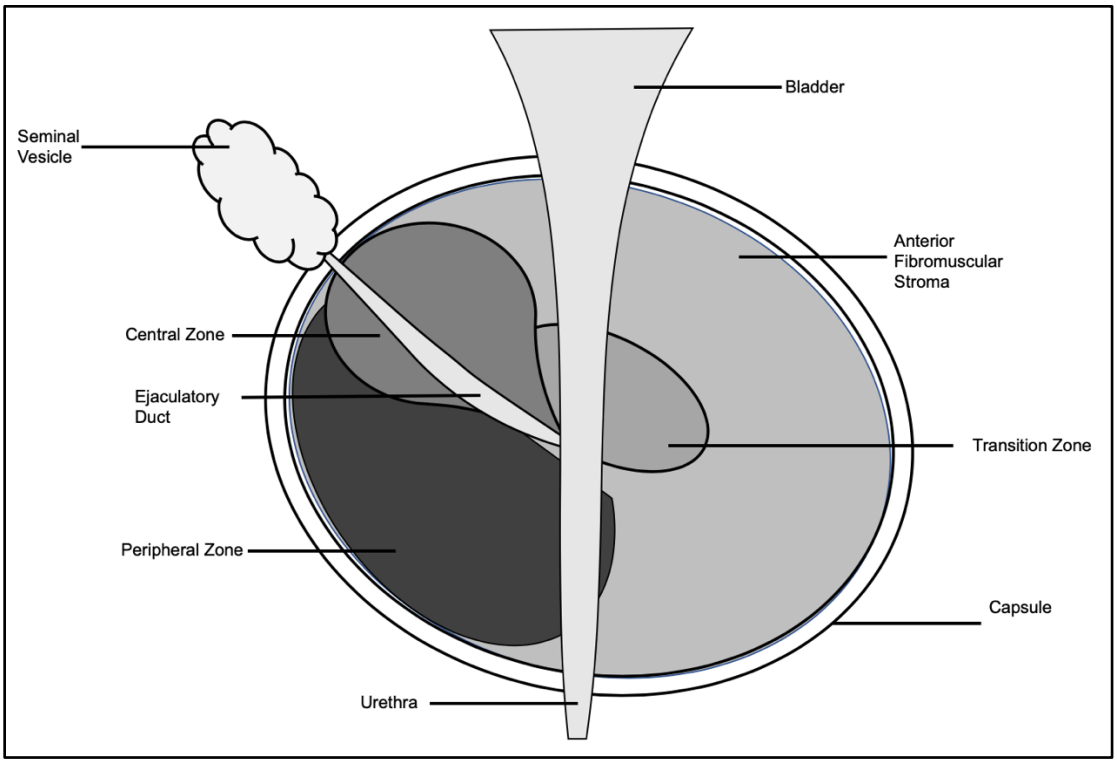


Figure 1. **Schematic of the human prostate anatomy**. The prostate is classically subdivided in to three zones. The peripheral zone is the largest zone of the prostate. The transition zone is the part of the prostate that surrounds the urethra. The central zone lies around the ejaculatory ducts and is a rare place for tumor development, however these cancers are typically highly aggressive in nature.

Prostate cancer is defined as the malignant, uncontrolled growth of cells within the prostate gland. Prostate cancer is the most common non-cutaneous cancer diagnosed in men with about 1 in 6 diagnosed with prostate cancer in their lifetime. Annually, there are approximately 175,000 new incidences of prostate cancer in the Unites States alone. About 1 in 41 men will die of prostate cancer and there are roughly 32,000 prostate cancer related deaths in the United States each year [13]. The vast majority of prostate cancers are classified as adenocarcinomas of the epithelial gland cells in the lumen of the prostate.

The exact cause of prostate cancer remains unknown, however there exists several factors that have been linked to an increase risk for the development of prostate cancer. The number one risk factor for prostate cancer is advanced age, with 6 in 10 incidences of prostate cancer diagnosed in men over 65. Familial history or African American ancestry are all risk factors associated with increased propensity to develop prostate cancer [14]. The presence of chronic inflammation in the prostate caused by a multitude of different factors such as environmental exposures, dietary factors, hormonal changes, or infections has also been linked prostate cancer development [15]. The presence of chronic inflammation may lead to cellular damage that increases the rate of mutation and transition of a normal cell to a cancer cell. A limited number of prostate cancers, roughly 5%, result from inherited mutations in genes that are involved in functions like DNA repair (BRCA2).

Although the exact pathway of prostate cancer progression remains under debate, there is classically considered to be three main stages: initiation,

progression, and metastasis **(Figure 2)**. The initiation step involves the loss of basal cells and the expansion of luminal cells in to the duct to form a hyperplastic lesion referred to as Prostatic Intraepithelial Neoplasia (PIN) [16]. While not conclusive, it is often considered that PIN represents a precursor to prostate cancer. This step has been linked to cellular processes such as chronic inflammation, oxidative or DNA damage, and telomere shortening. Certain genetic factors have also been associated with this stage such as Myc oncogene overexpression [17] and NKX3.1 prostate specific tumor suppressor, a negative regulator or epithelial growth, down-regulation [18]. Phosphatase and Tensin Homolog (PTEN) is a negative regulator of PI3k/Akt pathway and is frequently deleted tumor suppressor in prostate cancer with monoallelic loss in 60% of prostate cancers. Complete loss of PTEN is associated with castration resistance and metastasis [19]. The most common molecular subtype of prostate TMPRSS2:ERG gene fusion is seen in approximately half of prostate cancer patients [20]. ERG overexpression contributes to the development of castration resistance through the disruption of AR signaling [21].

The transition to progression stages and adenocarcinoma, involves continual expansion of the luminal cells in to the duct which typically is associated with re-activation of developmental signaling pathways like ERK/MAPK [22]. Eventually the cells expand completely out of the duct, develop castration-resistance characterized by lack of response to androgen ablation, and undergo metastasis after Epithelial to Mesenchymal transition. Dysregulation of EZH2 a histone methyltransferase that acts to silence many tumor suppressor

genes that limit prostate cell growth and proliferation is often associated with this process [23].

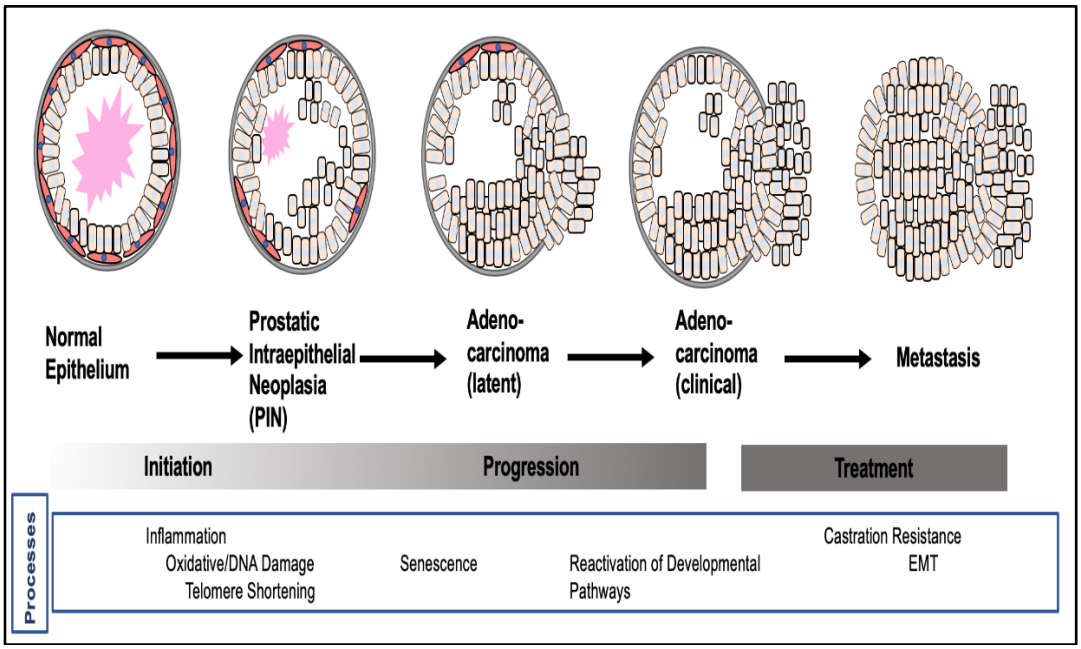


Figure 2. **Progression pathway for the human prostate cancer development.** The development of prostate cancer can crudely be defined in to the 3 sections: initiation, progression, and treatment stages. These stages of progression are highlighted above as well as the molecular processes that have been linked with playing a critical role in each stage. Each stage of progression involves the loss of some architecture of the normal epithelium leading to the eventual development of full blown metastatic prostate cancer. Adapted from Abate-Shen and Shen (2000).

The Gleason scoring system is a grading system that is used to calculate aggressiveness of prostate cancers. This system is a critical component for helping physicians choose suitable treatment options for individual patients based on the characteristics of the tumor. The Gleason scoring system involves a range from 1-5: 1 representing small, uniform glands, 2 representing more space, stroma between glands, 3 representing distinct infiltration of cells at gland margins, 4 representing irregular masses of neoplastic cells with few glands, and 5 describing poorly differentiated abnormal tissue. When a Gleason score is given to a patient two scores are provided to account for the fact that most prostate cancers are made up of cells that would fall in distinctive grades. The first score is considered the primary score and represents the score for the largest section of the tumor. The next largest area of the tumor is used to provide the secondary score. The summation of these two scores determines the Gleason score [24, 25].

A score of 6 or less in the Gleason system describes cancer cells that more closely resemble normal cells, are generally slow growing, and tend to remain localized. A score of 7 generally provides the patient with the idea that they are at a medium risk for aggressive cancer that depends on the breakdown of the scoring. If a tumor is given a score of 3+4 it is likely to remain in the prostate and have a more favorable outlook. A tumor with a score of 4+3 tend to be more aggressive and have an increased likelihood of spreading and further growth. In fact 4+3 cancers have been shown to have a 3 fold increase in

lethality over 3+4 cancers [26]. Anything that scores over 8 is considered to be a high-grade tumor that is likely highly aggressive and will spread quickly.

The gleason score is utilized to calculate the grade group for prostate cancer patients. Grade group 1 is a Gleason score of 6 or less and grade group 2 or 3 is a Gleason score of 7. Grade group 4 is a Gleason score of 8 and grade group 5 is a Gleason score of 9 or 10. The grade group and prostate specific antigen level test are used to stage the prostate cancer. The stage of the prostate cancer will dictate the treatment guidelines that will be followed clinically.

There are four unique stages in prostate cancer: Stage I, Stage II, Stage III, and Stage IV. Stage I prostate cancer is found only in the prostate. The cancer can be discovered by needle biopsy during tissue removal surgery. Stage I cancer may also be detected by digital rectal exam and found in one-half or less of one side of the prostate. Stage I prostate cancer is classified as grade group 1 and PSA level less than 10. Stage II can be subdivided into IIA, IIB, and IIC but the prostate cancer is still contained within the prostate. Stage IIA prostate cancers are grade group 1 and have PSA level of at least 10 but less than 20. Stage IIB are grade group 2 with a PSA level of less than 20 and the prostate cancer is in one or both sides of the prostate. Stage IIC prostate cancers are grade group 3 or 4 and PSA level less than 20. Stage III prostate cancer can be subdivided into IIIA, IIIB, and IIIC. Stage IIIA the PSA level is at least 20 and grade group 1, 2, 3, or 4. Stage IIIB the cancer has spread to seminal vesicles or nearby tissues or organs, the PSA can be any level, and grade group 1, 2, 3, or 4. Stage IIIC the PSA can be any level and the grade group is 5. Stage IV can be

subdivided into IVA and IVB. Stage IVA the prostate cancer has spread to local tissues and nearby lymph nodes and is grade group 1, 2, 3, 4, or 5. Stage IVB the cancer has spread to other parts of the body such as bones or distant lymph nodes [27].

1.1.2 Current Treatment Options

The treatment roadmap for patients with prostate cancer depends on the stage of the prostate cancer and the sequential progression of treatment options is highlighted in **Figure 3**. Standard treatment of stage I involves the use of active surveillance or watchful waiting [28]. This stage may also include treatment options such as high-intensity ultrasounds, radiation therapy, or radiopharmaceutical therapy to ablate the prostate cancer cells [29]. Patients whom previous treatments are unsuccessful or have a high risk of progression, often times radical prostatectomy to completely remove the prostate is performed followed by radiation therapy [30].

Stage II prostate cancer treatment plan may also include watchful waiting and active surveillance, however if the cancer begins to grow hormone therapy is often given. Similar to stage I, radical prostatectomy or internal therapy radiation may be necessary for stage II. Stage III prostate cancer treatment involves external radiation therapy with subsequent hormone therapy. The standard of care for stage IV prostate cancer is hormone therapy with or without chemotherapy.

Often times the above treatment regimen is successful and contributes greatly to the nearly 99% survival rate for prostate cancer patients, however, for patients with recurrent or metastatic castration resistant prostate cancer (mCRPC) androgen deprivation therapy (ADT) is the standard of care followed by chemotherapy if necessary. Since androgens and androgen signaling are the main contributor to prostate growth and homeostasis which becomes exacerbated during prostate cancer progression, androgen deprivation therapy's goal is to reduce the androgen receptor activity and level of androgens. ADT can be accomplished by physical or chemical castration, the use of androgen receptor antagonists, or luteinizing-hormone release agonists [31]. Biological therapy with sipuleucel-T may be an option for patients already treated with ADT.

More recently, there has been expanding interesting data that suggest the familiar standard of care chemotherapeutic for metastatic disease, docetaxel may be a significant option for patients with hormone-sensitive or earlier stage prostate cancer. For a long-time, docetaxel has been not used in this context because of potential toxicities associated with its use that have been deemed unnecessary to put this population of patients through. Therefore, docetaxel has been saved for patients with metastatic disease that have high tumor burden. However, in multiple studies docetaxel has proven beneficial in conjunction with ADT for localized prostate cancers. In the CHAARTED and STAMPEDE trials the addition of rounds of docetaxel improved overall survival of prostate cancer patients [32]. Lately, updated results from another study, GETUG-12, has

demonstrated that the addition of four rounds of docetaxel reduces the risk of relapse or death in men with high-risk localized disease [33].

1.1.2.1 After Androgen Deprivation Therapy

Unfortunately, for many patients ADT fails and the cancer becomes castration resistant. In such cases, chemotherapies like the aforementioned docetaxel and cabazitaxel are used as the standards of care for treatment of metastatic cancers and have been shown to improve patient survival [31]. Paclitaxel is not implicated for use in prostate cancer because of the dose-limiting toxicity peripheral neuropathy [34]. However, in the context of prostate cancer nearly 50% of all patients do not respond to taxanes and ultimately all develop resistance [35]. Hence, there still exists a tremendous need for new targets and drugs to sensitize these resistant cancers to current standard of care treatments.

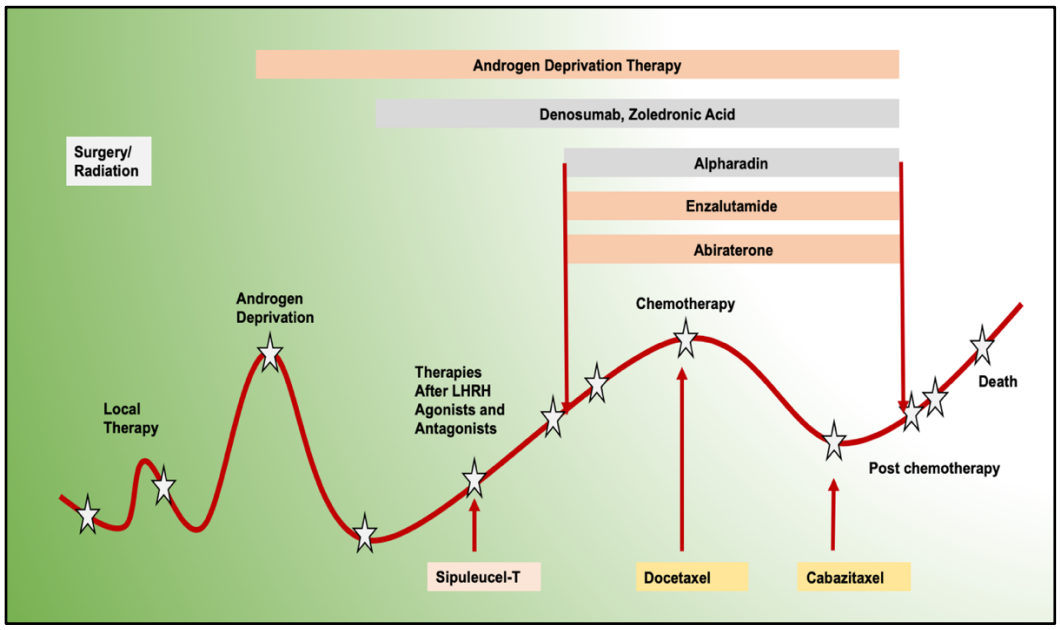


Figure 3. **Progression of treatments given to prostate cancer patients.** Curved red line represents increasing PSA level over time. In prostate cancer localized to the prostate, local therapy is applied which includes surveillance, waiting, or surgery. After progression of the cancer, androgen deprivation therapy is used to deplete the cancer cells of androgens required for growth and proliferation. If the prostate cancer progresses to a castration-resistant metastatic form, taxane chemotherapies are given to patients.

1.2 Docetaxel Resistance Mechanisms

Docetaxel, a taxane chemotherapeutic, has been a mainstay treatment for men with metastatic prostate cancer since 2004 and is still considered the standard of care for patients with late stage disease [36]. Docetaxel exerts its function by poisoning the mitotic spindle apparatus by binding beta-tubulin in microtubules, stabilizing the microtubule structures and preventing their dynamic polymerization and depolymerization [37]. As mentioned before docetaxel has been plagued by the fact that half of mCRPC patients do not respond to docetaxel and ultimately all patients develop resistance. The development of docetaxel resistance has been the limiting factor contributing to docetaxel only increasing overall patient survival 3 months over previous treatment regimens [38].

1.2.1 Proposed Mechanisms

There exists a multitude of docetaxel resistance mechanisms that have been reported that allow prostate cancer cells to escape the mitotic arrest induced by docetaxel treatment. The first identified mechanism is the overexpression of multi-drug resistance genes in prostate cancer. Specifically, MDR1/ABCB1 gene codes for the p-glycoprotein (P-gp) drug efflux pump which extrudes docetaxel from the cell and prevents its cytoplasmic accumulation [39]. Interestingly, P-gp expression is correlated with higher tumor grade and stage as well as increased prostate specific antigen (PSA) levels [40]. Alterations in tubulin expression have also been associated with docetaxel resistance in

prostate cancer. Elevated expression of βIII-tubulin has been demonstrated to lead to taxane resistance because of microtubules containing βIII-tubulin are less stable and have aberrant dynamicity that reduces the effectiveness of docetaxel to exert its mechanism of action [41]. Finally, defects in apoptotic pathway signaling and regulation have been increasingly implicated as a potential mechanism for docetaxel resistance. One such defect that is of particular interest to our lab is the overexpression of Inhibitor of Apoptosis Protein (IAP) Family members, particularly survivin, which leads to an imbalance of anti-apoptotic proteins to pro-apoptotic proteins resulting in chemoresistance and cancer progression [42]. As docetaxel exerts its cytotoxic effects by disrupting microtubule dynamics and promoting apoptosis through cell cycle arrest, survivin as a key molecular player in both processes is likely a critical component of the docetaxel resistant phenotype. Thus, targeting survivin may be a viable strategy for sensitizing resistant prostate cancer cells to docetaxel.

1.3 Survivin and IAP Family

1.3.1 IAP Protein Family

The IAP family members are historically described as the major regulators of the apoptosis pathway because of their ability to inhibit the cysteine proteases, caspases [43]. Recently, IAPs have become of increasing interest as potential biomarkers and therapeutic targets in cancer due to their dysregulation and overexpression being associated with poor clinical outcomes and chemotherapy resistance [44]. The IAP family consists of eight unique members: Neuronal

Apoptosis Inhibitory Protein (BIRC1), Cellular Inhibitor of Apoptosis Protein 1 (BIRC2), Cellular Inhibitor of Apoptosis Protein 2 (BIRC3), X-linked Inhibitor of Apoptosis Protein (BIRC4), Survivin (BIRC5), BIR Repeat-Containing Ubiquitin Conjugating Enzyme (BIRC6), Livin (BIRC7), and IAP-like Protein 2 (BIRC8) (**Table 1**). Each member of the family is characterized by the existence of 1 to 3 Baculovirus IAP Repeat (BIR) domains and the majority of the proteins also contain a carboxyl-terminal RING domain [45]. CIAP-1 and CIAP-2 are the only members that possess a caspase recruitment domain [46]. Although all members of the family contain a BIR domain thought to be necessary for binding caspases, XIAP is the only IAP protein shown to physically associate with caspases directly and inhibit their activity [47]. XIAP also contains an E3 ligase domain capable of ubiquitination of caspases to promote their degradation and inactivation [48].

1.3.2 Apoptosis Pathway

As mentioned, the IAP proteins are critical regulators of the intrinsic and extrinsic programmed cell death pathways. In the intrinsic apoptosis pathway, non-receptor mediated stimuli that negatively affect the cell such as growth factor withdrawal, hypoxia, or DNA damage is detected by the cell [49]. These stimuli result in changes to the inner mitochondrial membrane that lead to the opening of the mitochondrial permeability transition pore and the subsequent release of pro-apoptotic proteins cytochrome c and Smac/DIABLO [50]. Cytochrome c binds to Apaf-1, activates it, and complexes with pro-caspase 9 to form what is known as the apoptosome [51]. The apoptosome activates caspase 9 which cleaves and

activates caspase 3 to start the execution pathway. Smac/DIABLO is known to bind and suppress the activity of IAP family members [52].

The extrinsic pathway involves receptor-mediated interactions at the cell membrane involving death ligands such as TNF-α, Fas ligand, or Targeting TNF-related apoptosis-inducing ligand (TRAIL) [53, 54]. Upon binding of a death ligand to a death receptor, the receptors oligomerize bringing their death domains in contact which results in the recruitment of specialized adaptor proteins. These adaptors provide the requisite scaffolding for procaspase 8 binding to form the death inducing signaling complex (DISC) [55]. Activated caspase 8 can then cleave caspase 3 to begin the execution pathway. C-FLIP is a master anti-apoptotic regulator that can be recruited to the DISC complex and inhibit caspase 8 activation [56]. The extrinsic pathway connects to the intrinsic pathway through the cleaving of a protein BID to t-BID, which translocate to the mitochondria to promote outer mitochondrial membrane permeabilization [57].

1.3.3 Survivin Structure and Function

Survivin (also called BIRC5) represents the smallest member of the IAP family with a molecular weight of 16.5 kDa [58]. Survivin consists of 142 amino acid residues with a single zinc finger fold Baculovirus Repeat (BIR) domain, a dimerization domain at two different locations in the linear sequence, and a C-terminal domain for protein-protein interaction and nuclear export (**Figure 4A and 4B**). Survivin exists as a stable bow-tie-shaped homo-dimer (**Figure 4C**) formed through interactions of the N-terminal region, linker region, and the N-

terminus of alpha helix four [59]. The homo-dimeric interface is mostly hydrophobic with greater than 75% of the residues being nonpolar [60]. The buried accessible area in the interface of a monomeric survivin is 550 $Å^2$ and occupies only 6% of the total accessible area of a monomer (9,044 $Å^2$) compared with ~20% in most other dimeric or oligomeric proteins [61].

Table 1. **Members of the IAP family.**

IAP Member	Abbreviation	Mol. Wt.	Reference
Neuronal apoptosis inhibitory protein	NAIP/BIRC1	160 kDa	[62]
Cellular Inhibitor of Apoptosis protein 1	c-IAP1/BIRC2	69 kDa	[63]
Cellular Inhibitor of Apoptosis protein 2	c-IAP2/BIRC3	68 kDa	[63]
X-linked Inhibitor of Apoptosis protein	XIAP/BIRC4	55 kDa	[64]
Survivin	Survivin/BIRC5	16.5 kDa	[65]
BIR-repeat-containing ubiquitin conjugating enzyme	BRUCE/BIRC6	528 kDa	[66]
Melanoma IAP/Livin	ML-IAP/livin/BIRC7	33 kDa	[67]
IAP-like protein 2	ILP2/BIRC8	27 kDa	[68]

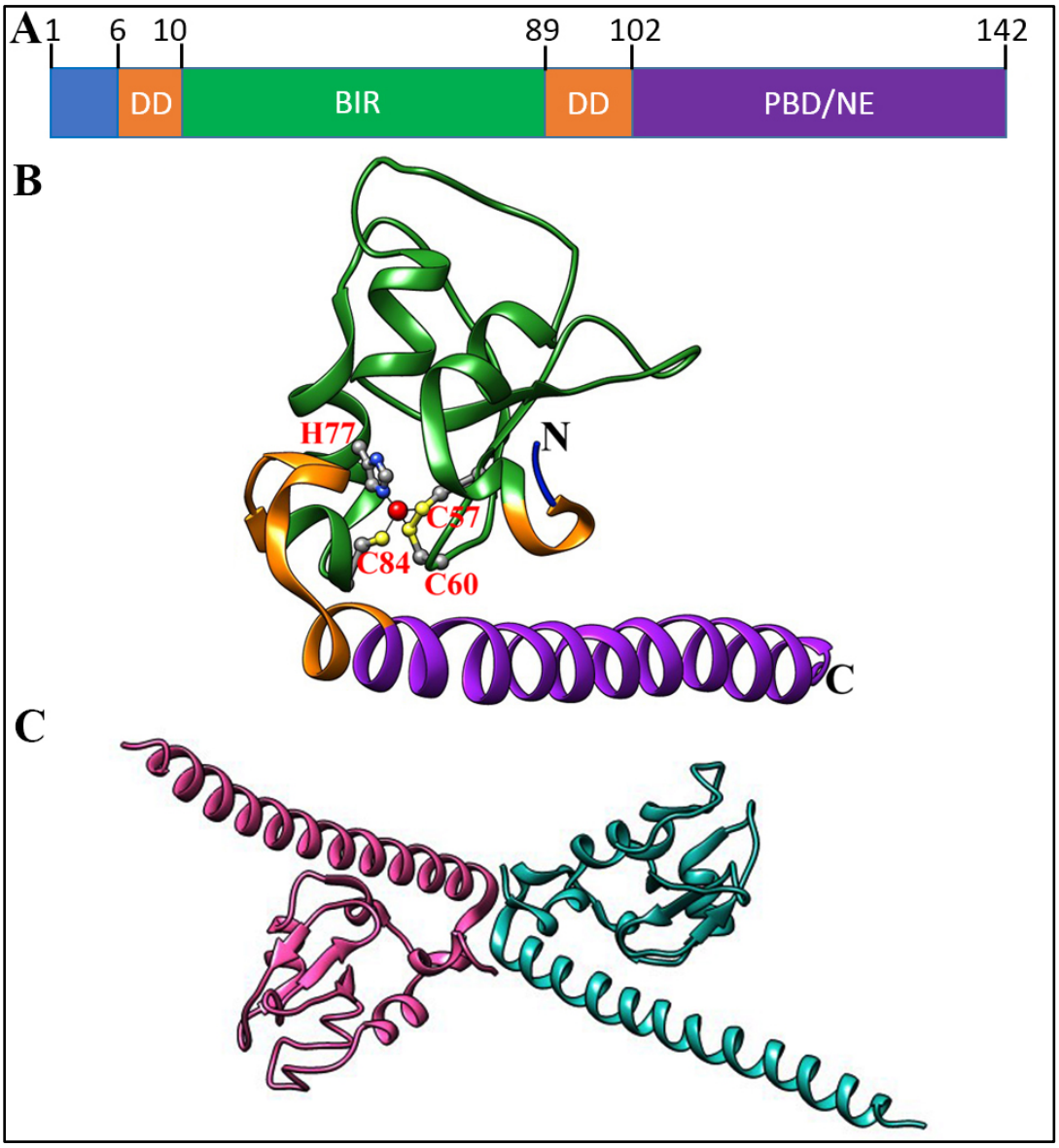


Figure 4. **Structure of survivin**. **A**. Schematic linear structure of survivin with domains highlighted in different colors. DD, dimerization domain; BIR, Baculovirus Repeat domain; PBD, protein-binding domain; NE, nuclear export. **B**. Atomic structure of survivin (PDB 1F3H) with domains color in correspondence with that shown in the linear structure in panel A. The zinc finger residues (Cys^{57}, Cys^{60}, His^{77}, and Cys^{84}) are shown in ball and stick symbols with the zinc ion shown as a red ball. **C**. Atomic structure of dimeric survivin (PDB 1F3H) with one subunit shown in pink and the other in blue. From *Peery and Zhang* (2017).

Ectopic over-expression of survivin inhibits both extrinsic and intrinsic apoptosis pathways in cell lines and animal models [69-72]. Survivin's mechanism of inhibition of apoptosis remains elusive, however several possibilities have been proposed (**Figure 5**). Survivin has been suggested to directly bind to and inhibit caspase 3 and 7 while contradictory evidence exists that bring this observation under scrutiny [73, 74]. Although myc-tagged survivin in HEK293 cells co-immunoprecipitated with active caspase 3 and 7, survivin seemingly lacks the additional structural moieties such as the BIR2 domain that have been demonstrated to be necessary for caspase 3 and 7 binding by other IAP family members [75, 76]. It has also been suggested that survivin can bind to caspase 9 and inhibit its activation [77]. However, it was shown later using purified proteins that survivin alone does not bind to caspase 9 and it may require the presence of hepatitis B X-interacting protein (HBXIP) to bind to and inhibit caspase 9. The interaction between survivin and HBXIP may be responsible for binding to pro-caspase 9 to prevent its recruitment to the apoptosome and, thus, inhibit its activation [78]. It has also been postulated that an IAP-IAP complex between survivin and X-linked IAP (XIAP) may form to stabilize XIAP and lead to inhibition of caspase 9 [79]. Finally, survivin may prevent the induction of apoptosis by interaction with intermediate apoptotic proteins to indirectly halt caspase activation. SMAC/DIABLO is a proapoptotic protein that promotes cytochrome c dependent apoptosis by binding and antagonizing IAPs [80]. Survivin can bind SMAC/DIABLO and prevent SMAC/DIABLO inhibition of caspases [81]. Survivin has been shown to co-localize with SMAC/DIABLO and

disruption of their physical association induces apoptosis [82]. Thus, it is possible that survivin inhibits apoptosis via interaction with multiple proteins in the apoptosis pathway.

In addition to the inhibition of apoptosis, survivin acts as a key mitotic regulator (**Figure 5**) and is essential for proper completion of mitosis and cell division [83]. Survivin expression is tightly regulated during cell cycle progression, peaking in the G2/M phase [84]. During mitosis, survivin localizes and interacts with microtubules to regulate microtubule formation during cell division by altering microtubule dynamics and nucleation [85]. Additionally, survivin is a critical component of the chromosomal passenger complex (CPC) that also consists of INCENP, Borealis, and Aurora B Kinase [86]. INCENP acts as a scaffold to stabilize the entire complex, while Borealis promotes the attachment of survivin to the complex [87]. Survivin appears to function in targeting of the CPC to the centromere and midbody during mitosis. The CPC is a critical regulator of several mitotic events [88] and, when localized to the midbody, it facilitates proper chromosome alignment during metaphase [89]. The CPC is also vital for correction of chromosome-microtubule attachment errors [90] and activation of the spindle assembly checkpoint [91].

It has also been suggested survivin may be functioning to promote tumor progression via angiogenesis and metastasis. By an incomplete mechanism, survivin appears to upregulate Vascular Endothelial Growth Factor (VEGF) in endothelial cells to promote their proliferation. It is likely this involves positive cell signaling via the PI3K/Akt pathway to enhance B-catenin dependent transcription

of VEGF [92]. In the context of cancers such as glioma and breast, knocking down survivin decreases angiogenesis [93, 94]. There is growing evidence that survivin may contribute to metastasis of cancer cells via coordination with another Inhibitor of Apoptosis member, XIAP. In a mouse breast cancer model, survivin and XIAP worked in conjunction to promote cell invasion and metastatic dissemination. The promotion of these aggressive cancer trademarks appears to likely be caused by an increase in NF-κB and cell motility kinases activation [95]. Finally, survivin overexpression has been associated with increased metastasis in patient studies of colorectal and prostate cancers [96, 97]. As more research follows, it is becoming clearer that survivin has an ever-expanding role outside of its classical inhibition of apoptosis that contributes to other classical hallmarks of cancer.

1.3.4 Survivin Regulation

The regulation of the survivin gene is highly dynamic and involves key players in cell survival, cell proliferation, and developmental signaling pathways. At the transcriptional level, survivin is negatively regulated by several tumor suppressor genes such as PTEN [98] and p53 [99]. In normal cells, the p53 target protein p21 is primarily responsible for maintenance of the low survivin expression pattern [100]. Notch 2 signaling has also been shown to suppress survivin transcription [101]. Egr-1 and KLF4 transcription factors are capable of binding survivin promoter and repressing its expression [102, 103]. Finally, in prostate epithelial cells TGF-β has been shown to downregulate survivin

expression via a Smad2/3 hypophosphorylation of Rb protein dependent pathway [104]. Deregulation of these negative regulators and their signaling pathways may result in the overexpression of survivin seen in most solid cancers.

Survivin expression is positively regulated by several pro-oncogenic transcription factors which contain putative binding sites in the survivin promoter, such as STAT3, Sp1, NF-kB, and E2F1 [105]. The most important of these transcription factors in relation to survivin expression appears to be specific protein 1 (Sp1). In the survivin proximal promoter, there are several G/C rich Sp-binding sites and two have been identified as being essential for sustaining basal survivin levels [106]. Wnt/β-catenin signaling is known to upregulate survivin expression in colorectal and melanoma cancer cells [107, 108]. In breast cancer, Stat3 has been demonstrated to be a critical driver of survivin transcription [109]. In liver hepatocytes, survivin is regulated by NF-kB signaling [110]. Finally, in prostate cancer cells the androgen receptor upregulates survivin expression [111].

Survivin undergoes several post-translational modifications that are important for the regulation of its expression and its activities in the cell. During mitosis, cell division cycle protein 2 (Cdc2) phosphorylates survivin at threonine 34, which increases the stability of survivin by reducing its proteasomal degradation. This phosphorylation event may also be important for survivin's physical interaction with XIAP, as Threonine 34 lies within the single BIR domain of survivin [112]. Thus, this modification may boost its cytoprotective effect in

cancer cells as well. Survivin phosphorylation at threonine 117 by Aurora-B kinase is critical for survivin's localization to chromosomes and microtubules during mitosis [113]. Protein kinase A phosphorylation of survivin at serine 20 interferes with survivin's binding to XIAP and diminishes its anti-apoptotic role in the cell [114]. CREB-binding protein acetylation of survivin on lysine 129 is critical for the nuclear localization of survivin. Deacetylation at this site is responsible for the accumulation of survivin monomers that complex with CRM1 nuclear export protein to shuttle survivin out of the nucleus [115]. Overall, the dynamic status of these post-translational modifications of survivin are crucial in promoting survivin's functional roles and its localization in the cell.

The bifunctionality of survivin can be attributed to its subcellular distribution to different pools in the cell. Survivin has classically been demonstrated to exist in pools in the cytoplasm, nucleus, and mitochondria. The respective distribution of survivin into each of these separate pools appears to be cell type specific. In the mitochondrial and cytoplasmic pools, survivin acts in its anti-apoptotic capacity where it complexes with other IAPs to inhibit caspase activation and activity. As previously mentioned, in the nuclear pool survivin acts in the CPC to position and align chromosomes during mitosis.

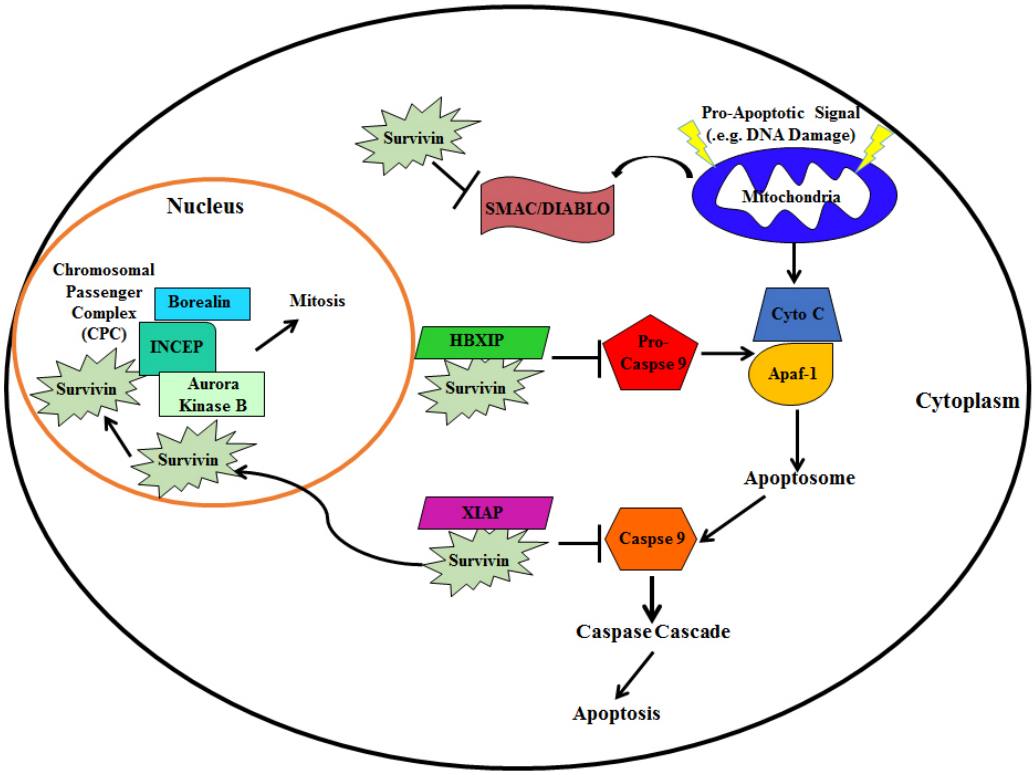


Figure 5. **Participation of survivin in regulating apoptosis and cell cycle progression.** Survivin regulates cell cycle progression by binding to the chromosomal passenger complex in the nucleus and helping to orient and align chromosomes during metaphase. Survivin also regulates apoptosis by binding to and inhibiting SMAC/DIABLO and complexing with other proteins like XIAP and HBXIP to prevent caspase activity and the eventual caspase cascade execution pathway of apoptosis. From *Peery and Zhang* (2017).

1.4 Drug Discovery Cycle

The process of drug discovery and development is long, arduous, and expensive process with many cyclical iterations. The general overview of the drug discovery cycle is highlighted in **Figure 6**. The process begins with the identification of a druggable biological target with an implication in human disease followed by the development of assays to assess the in vitro activity of the target. The next step is an iterative cyclical process that begins with the screening of libraries of compounds. This is followed by in vitro high throughput screening and cell-based assays to identify compounds and cells response to them. Many times this is often accompanied with secondary screening of promising candidates *in vivo* for assessment of bioavailability, safety, and efficacy. This cyclical process eventually yields a primary hit for future analysis. The compound hit is then typically utilized in structure activity relationship (SAR) assays to synthesize analogues. The analogues are tested in cell-based assays to develop a primary lead. The final steps of the drug discovery process involves another round of iterative cyclical steps in which optimized leads are tested in vivo for efficacy, safety, and pharmacodynamics and pharmacokinetics properties. After many repeated cycles of this process, the goal is to have established a candidate drug with a strong safety profile for clinical development. At this stage the goal is to have a portfolio for this compound ready to be approved for Phase I clinical trials [116].

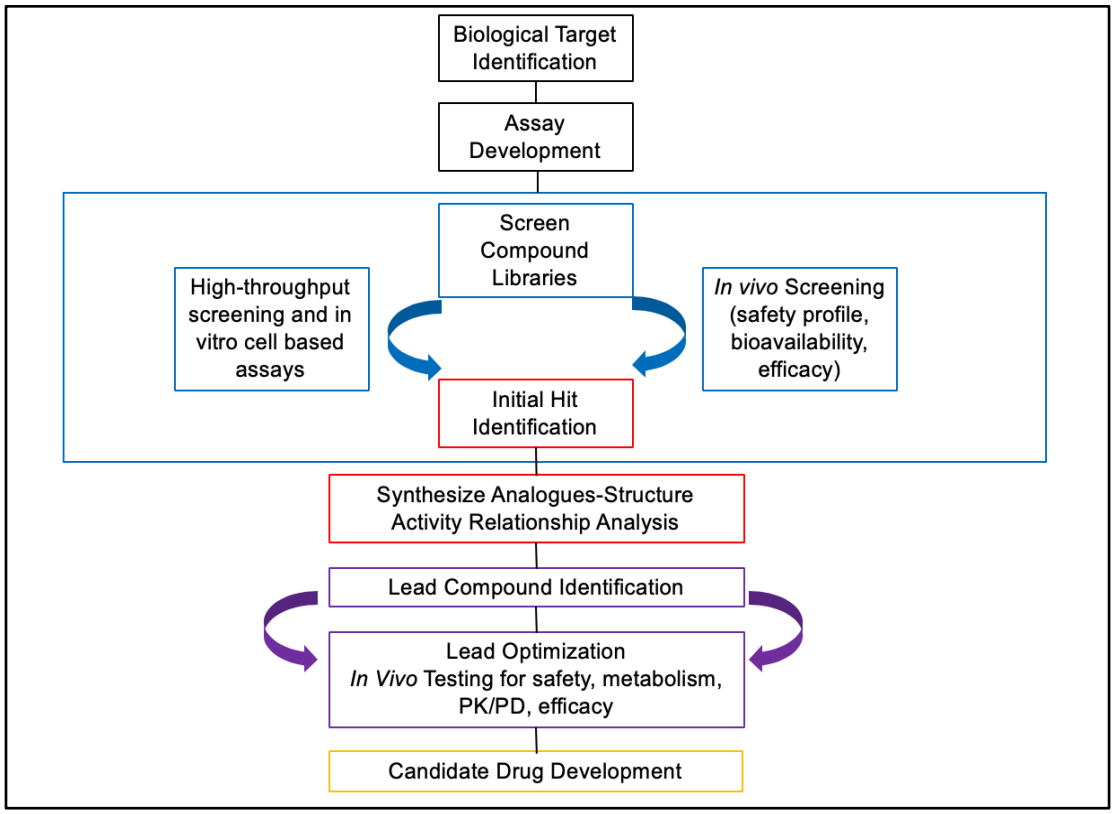


Figure 6. **Drug Discovery Cycle.** The development of small molecule inhibitors is a long cyclical process that involves identification of a biological target and screening of large libraries of compounds. After rounds of characterization assays, an initial hit is taken to structure activity relationship analyses. A lead compound is identified and goes through a series of *in vivo* optimization assays before a portfolio is built for candidate drug development in Phase 1 clinical trials. Adapted from Cohrt (2018).

1.5 Survivin as a Target for Drug Discovery

For over a decade, survivin has drawn a considerable attention as a potential novel cancer target in a variety of human cancers. The attractiveness of survivin as a potential cancer drug target has been in large part due to its dramatic dysregulation of expression between normal adult tissue and most cancers. Survivin is expressed to a high extent in fetal tissues [117], but is undetectable in most normal adult tissues [118]. Interestingly, survivin is overexpressed in almost all human cancers including cancers of lung, breast, colon, stomach, esophagus, pancreas, prostate, liver, and ovary [119]. Survivin has been consistently demonstrated to be an important contributor to both radiotherapy [120] and chemotherapy resistance [121, 122]. High level of survivin expression is predictive of poor clinical outcome [123] and correlates with tumor relapse [124]. Expressing recombinant dominant negative survivin Thr34A prevented its association with XIAP and caspase 9 and produced pro-apoptotic and anti-proliferative effects on human cancer cells and suppressed tumor growth in vivo [125-127]. Cleavage of survivin mRNA by ribozyme increased cell death by caspase dependent apoptosis [128]. Down regulation of survivin expression by antisense oligonucleotides [129] and siRNAs inhibited cancer cell proliferation and increased chemosensitivity [130]. These studies using molecular probes along with the expression profile of survivin in cancer have established survivin as a target for anticancer drug discovery. Survivin's dual role in cancer cell survival as well as its contribution to cancer hallmarks angiogenesis and metastasis also serve to highlight its attractiveness as an anti-cancer agent [131].

1.5.1 Therapeutics Targeting Survivin

Despite the promise and interest in survivin as a target for anticancer drug discovery, there presently exists a small portfolio of anti-survivin agents, which can generally be classified in to three broad categories of inhibitors targeting (1) regulators of survivin expression, (2) survivin interaction with ligand proteins, and (3) survivin homo-dimerization. In addition, survivin has also been considered and tested as a cancer vaccine for immunotherapy.

1.5.1.1 Inhibitors Targeting Survivin Expression

Since survivin has no known enzymatic activities and it is considered "undruggable", the initial effort of targeting survivin was not on the survivin protein itself, but rather on inhibiting survivin expression to avoid targeting survivin protein directly. This approach includes the use of antisense oligonucleotides and small molecule inhibitors.

1.5.1.1A Antisense Oligonucleotides

Antisense oligonucleotides such as LY2181308 and SPC3042/EZN-3042 (**Table 2**) have been developed to inhibit survivin expression as anticancer therapeutics. These oligonucleotides have been tested in clinical studies with mixed findings. LY2181308 is a 2'-O-methoxymethyl-modified single strand antisense oligonucleotide targeting survivin mRNA to limit survivin expression developed by Eli Lilly [132]. LY2181308 inhibited the expression of survivin gene at both mRNA and protein levels in a panel of cell lines and it significantly

inhibited growth of human tumor xenografts [133]. The positive pre-clinical activity of LY2181308 led to its clinical testing as a single agent and in combination with other chemotherapeutics. However, the outcome of these clinical studies are mixed. LY2181308 was well tolerated as a single agent and did not appear to cause additional toxicity to cytarabine and idarubicin in refractory or relapsed Acute Myeloid Leukemia patients [134]. With this cohort of 16 AML patients, it appeared that the combination of LY218308 with cytarabine and idarubicin showed added benefits. LY218308 also inhibited survivin expression in patients with high survivin level although the correlation between survivin inhibition and response was not studied. It is also noteworthy that the cohort size in this study was small and a bigger cohort size is needed to validate the findings on added benefits.

The findings from clinical study of LY218308 on solid tumors are not encouraging. Neither the phase I trial of LY218308 as a single agent for solid tumors [135] or in combination with docetaxel/prednisone in a phase II trial for castration-resistant prostate cancers [136] showed any benefit of LY2181308. The authors noted that lack of response might be due to the lack of survivin inhibition by LY2181308 in these solid tumors although survivin expression was not measured in these studies and they were unable to achieve pharmacokinetic levels necessary for survivin inhibition required. SPC3042 is a locked antisense oligonucleotide that targets the stop codon of the open reading frame in exon 4 of the survivin transcript and was under preclinical development by Santaris Pharma [137]. SPC3042 displayed improved potency as compared to

LY2181308. However, unlike LY2181308, SPC3042 had a significant effect also on the mRNA and protein levels of Bcl-2 in addition to that of survivin. Down regulation of survivin expression by SPC3042 sensitized PC-3 prostate cancer xenograft to taxol treatment in vivo. In 2006, Santaris Pharmaceuticals licensed the developmental rights of SPC3042 to Enzon-Pharmaceuticals and it was rebranded as EZN- 3042. In primary Acute Lymphoblastic Leukemia cells, EZN-3042 synergized with chemotherapy and eliminated ALL cells in vitro [138]. EZN-3042 also demonstrated success in vivo as it achieved ~60% down regulation of survivin mRNA in Calu-6 lung xenografts and ~40% tumor growth inhibition. In combination with paclitaxel, EZN-3042 achieved >80% tumor growth inhibition [139]. However, the phase I trial of EZN-3042 was terminated since EZN-3042 produced several dose-limiting toxicities and it was unable to be safely administered with other chemotherapeutics [140]. As such, further clinical development of this oligonucleotide has been halted by Enzon Pharmaceuticals.

In summary, the success in clinical trials has been limited by targeting survivin expression using antisense oligonucleotides, particularly for solid tumors. The lack of response in solid tumors suggests that this strategy may have limited potential in treating the difficult-to-treat and aggressive solid tumors. One contributing factor to the ineffectiveness of antisense oligonucleotides may be the intrinsic disadvantages of oligonucleotides including stability and availability issues. These disadvantages may also account for the dose limiting toxicities that have been evident in previous trials and the currently limited portfolio of FDA-approved antisense oligonucleotide therapeutics.

Table 2. **Antisense oligonucleotides suppressing survivin expression.**

Name	Sequence	Remark	Reference
LY2181308	5'-TGTGCTATTCTGTGAATT-3'	In-vitro IC50 of 10-100 nM in inhibiting survivin expression. Lack of efficacy in phase II trials as a single agent or in combination with docetaxel	[132], [133], [134], [135], [136]
EZN-3042	5'-CTCAatccatggCAGc-3'	In-vitro IC50 of 5 nM in inhibiting survivin expression and IC50 of 5-40 μM in cell killing. Phase I trial terminated due to dose-limiting toxicities	[138], [139], [140]

1.5.1.1B Small Molecule Inhibitors

In addition to antisense oligonucleotides, small molecule inhibitors (**Table 3**) have also been developed by targeting transcription of the survivin gene. These inhibitors include YM155 and EM-1421, which have been tested in clinical studies, and other compounds such as SF002-96-1 and FL118, which are still at the pre-clinical stage. YM155 (sepantronium bromide) is the first small molecule inhibitor believed to be targeting the expression of survivin, identified via high throughput screening (HTS) using a survivin-promoter-luciferase reporter assay aiming to identify small molecule inhibitors that may bind to the promoter sequence of the survivin gene [141]. YM155 potently inhibited survivin-promoter-driven luciferase expression without effect on the expression of other antiapoptotic proteins. It also effectively inhibited growth of human prostate PC3 ectopic xenograft tumors. In a follow-up study, YM155 induced cell death with an average IC50 of 15 nM in a panel of 119 human cancer cell lines [142]. Continuous 3- or 7- day YM155 infusion in xenograft models also showed significant tumor suppressing activity on different tumors including melanoma and cancers of breast, lung, and bladder without significant toxicity as indicated by little body weight loss.

Due to the promise of YM155 in cell-based and preclinical studies, it has been investigated in clinical trials as a single agent and in combination with other anticancer therapies [143]. While YM155 is well tolerated with a MTD of 4.8 mg/m^2 and it has shown some efficacy against blood cancers, mixed results were generated when tested against various solid tumors with modest efficacy at

best for some tumors [144]. For example, no improvement in response rate was observed for non-small cell lung cancer patients in multiple phase II trials of YM155 as a single agent and in combination with carboplatin and paclitaxel [145, 146] while a phase II trial of castration resistant prostate cancers (CRPC) showed modest activity with 25% of patients displaying prolonged stable disease [147]. While the modest successes in clinical studies suggest that targeting survivin may help identify a novel approach of cancer treatments, successful clinical development of YM155 is now questionable. Additionally, one major problem with YM155 is that it cannot be used for bolus dosing and has to be continuously infused 24 hours a day in 3- or 7-day dosing cycles. Currently, there are no ongoing clinical trials with YM155. Since the discovery of YM155, there have been large undertakings to determine its precise mechanism of action. While it has been shown to inhibit survivin transcription, it does not work via binding to survivin promoter sequence as anticipated but rather by inhibiting the survivin upstream transcription factor Sp1 [148]. Evidence also exists to suggest that YM155 may inhibit survivin expression by disrupting interleukin enhancer-binding protein factor 3 and $p54^{nrb}$ complex, a critical complex for transcription of survivin [149]. There is also compelling evidence to suggest that YM155 may be a DNA damage-inducing agent [150] and its inhibition of survivin expression may be secondary to this event. Considering that Sp1, the target of YM155, is a ubiquitous transcription factor and that its inhibition of survivin expression may be a secondary event to DNA damages, designating YM155 as a survivin inhibitor is inappropriate and misleading.

EM-1421 (terameprocol) is another small molecule transcriptional repressor of the survivin gene under development as survivin inhibitor by Erimos Pharmaceuticals that acts to inhibit the ubiquitous transcription factor Sp1 [151]. In addition to inhibiting survivin expression, EM-1421 has been shown to also inhibit the expression of cdc2, another Sp1-controlled gene [152]. EM-1421 induced G2 cell cycle arrest in a variety of solid tumor cell lines [153] and systemic treatment with EM-1421 suppressed the growth of human tumor xenografts including breast, prostate, colorectal, and liver cancers [154]. Using clonogenic survival assays, it was found that EM-1421 was able to induce radio sensitization in non-small cell lung cancer cells [155]. Similar as YM155, EM-1421 has been tested in clinical trials. For advanced leukemia patients, EM-1421 demonstrated a safe profile and showed partial responses in a few patients with CML or AML in a phase I study [156]. However, in a phase I study for high grade gliomas, EM-1421 did not display any response but stable disease was observed in 32% of patients [157]. Most recently, the safety profile of EM-1421 was tested as a vaginal ointment for treatment of Cervical Intraepithelial Neoplasia caused by human papilloma virus [158] in a Phase I/II trial using healthy adult women. The ointment with 1% and 2% EM-1421 showed a promising safety profile with no severe adverse effects in healthy subjects.

SF002-96-1 is a natural drimane sesquiterpene lactone isolated from *Aspergillus* that was identified to potentially inhibit survivin promoter activity using survivin-promoter-driven reporter assay in Colo320 cells [159], similarly as described above for identification of YM155. SF002-96-1 decreased Colo320 cell

viability and induced apoptosis as evidenced by increased caspase 3 activity and cleaved chromosomal DNA by DAPI staining. Further studies using promoter-reporter assay, SF002-96-1 was shown to inhibit the activity of transcription factors STAT3 and NF-κB but not TCF/β-catenin, which all regulates survivin transcription, and also decreased survivin mRNA and protein levels. Thus, SF002-96-1 may inhibit survivin expression by inhibiting its upstream transcription factors such as STAT3 and NF-κB. As with other nonspecific strategies, unexpected dose-limiting toxicities may result from the disruption of other genes besides survivin, which may limit the utility of SF002-96-1 going forward.

FL118 is a nonselective small molecule inhibitor of survivin expression that structurally resembles the topoisomerase I inhibitor, irinotecan. FL118 appears to inhibit survivin expression by disrupting survivin promoter activity. However, FL118 also down regulates the expression of Mcl-1, XIAP, and cIAP2. Strikingly, FL118 displayed greater anti-tumor activity in squamous cell carcinoma and colon cancer cell line-derived xenografts, as well as a patient-derived head and neck cancer xenograft as compared to leading first line chemotherapeutics [160]. A detailed molecular mechanism of FL118 action is currently lacking but warranted with the promising in-vivo data. However, based on the studies of YM155, it is tempting to speculate that FL118 may also inhibit upstream transcription factors that regulate survivin expression. It may also be possible that the effect of FL118 on survivin expression is a secondary effect of DNA damage potentially induced by FL118 inhibition of topoisomerase (see

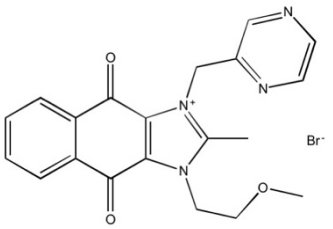
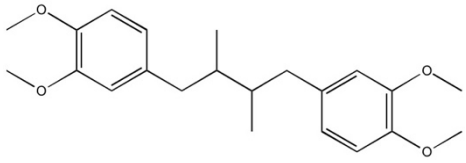
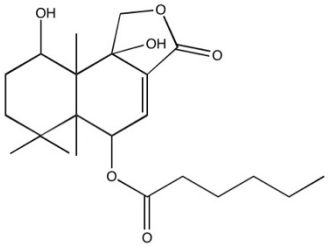
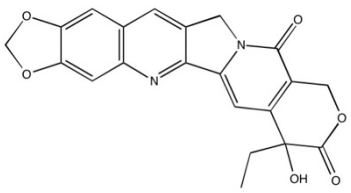
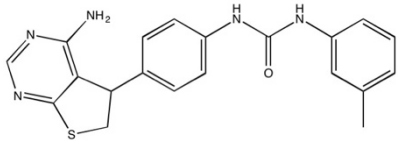
discussion above on YM155). GDP366 is a small molecule compound that has been found to reduce the mRNA and protein levels of both survivin and oncoprotein Op18. Treatment with GDP366 inhibits cancer cell proliferation both in vitro and in vivo [161]. Little data are available to evaluate GDP366 and more mechanistic studies are warranted to elucidate the mechanism of action by which GDP366 produces its effects on survivin expression and its potential as a cancer therapeutics.

While YM155 and EM-1421 have been tested in clinical trials as “survivin transcription inhibitors”, the most common issues with all these inhibitors are that they are not selectively targeting survivin expression. Most of these inhibitors act on one or more upstream transcription factors that regulate the expression of survivin as well as many other downstream genes. Effectively, these inhibitors are not survivin inhibitors but rather transcription factor inhibitors. The lack of specificity of these inhibitors in targeting survivin transcription as identified using survivin promoter activity assay and designating these compounds as survivin inhibitors are indeed troubling.

Although both YM155 and EM-1421 have generally been tolerated in clinical trials despite the fact that they both inhibit a ubiquitous transcription factor Sp1, their limited clinical efficacies suggest that targeting the survivin upstream transcription factors such as Sp1 to limit survivin expression may unlikely represent an appropriate strategy to target survivin for therapeutic development. It is also unclear if the other downstream target genes of these transcription factors may work against the inhibition of survivin expression in suppressing

cancers in the clinical setting. These findings also indicate that targeting Sp1 may not lead to effective cancer therapeutics for development.

Table 3. **Small molecule inhibitors suppressing survivin expression.**

Name	Structure	Remark	Reference
YM155		Inhibitor of Sp1. In-vitro IC50 of ~15 nM. Phase I and II trials with modest efficacy.	[141], [142]
EM-1421		Inhibitor of Sp1. In-vitro IC50 of 5-40 μM. Phase I and II trials with partial response.	[151], [152], [153], [154], [155]
SF002-96-1		Inhibitor of STAT3 and NF-κB In-vitro IC50 of 3.4 μM in inhibiting survivin promoter activity.	[159]
FL118		In-vitro IC50 of 6-8 nM In-vivo xenograft models of colon and head and neck cancers	[160]
GDP366		In-vitro IC50 of 1 μM (colon cancer cell line) In-vivo xenograft model of colon cancer	[161]

1.5.1.2 Inhibitors Targeting Survivin Interaction with Other Proteins

Since survivin is known to work by binding to and interacting with many important cellular proteins, there have been various efforts in targeting survivin interaction with its binding partners (**Table 4**). The first in this category of inhibitors is called shepherdin, a peptide with a sequence (K^{79}H SPGCAFL^{87}) of survivin that inhibits Hsp90 interaction with survivin and destabilizes survivin [162]. Tagging the amino terminus of shepherdin to either helix III of the Antennapedia or HIV-/Tat sequence led to a cell permeable shepherdin that was able to accumulate in cells and inhibit survival of HeLa, PC3, and DU145 cells by inducing apoptosis without apparent effect on normal cells. The cell permeable shepherdin also effectively inhibited PC3 xenograft growth in vivo. Because shepherdin binds to Hsp90 and may destabilize many proteins including survivin, the cell death induced by shepherdin could be due to multiple factors regulated by Hsp90. It would be of interest to determine if a peptide with a sequence from Hsp90 that inhibits Hsp90 binding to survivin has similar effects as shepherdin.

In another study searching for peptide mimetics interacting with survivin using yeast two-hybrid system, a peptide derived from heavy chain 1 of ferritin was identified to interact with survivin [163]. Interestingly, this peptide, when cloned into a disabled thioredoxin for purification of recombinant protein, was able to bind to survivin and suppress survivin expression and induce apoptosis in breast and glioma cancer cells. The reduced survivin expression appears to be proteasome dependent. However, these authors have also shown that the full-length heavy chain 1 of ferritin does not interact with survivin. Thus, the peptide-

induced survivin loss may not be via inhibition of survivin interaction with ferritin. It remains to be determined how this peptide derived from ferritin would induce loss of survivin and induce apoptosis of cancer cells. Using a shape-based structural screening for the second mitochondria-derived activator of caspase (SMAC) mimetic that would inhibit interaction between SMAC and IAP proteins, a novel hit compound, UC-112, was identified that significantly activate caspases in melanoma and prostate cancer cell lines [164]. Continuous treatment of A375 melanoma xenograft tumors with UC- 112 for 3 weeks significantly inhibited tumor growth in a dose-dependent manner with little reduction in the body weight of the mice. Interestingly, UC-112 dose-dependently inhibited survivin expression as well as expression of other IAPs albeit to a less extent. Currently, it is unknown if UC-112 disrupts the interaction between SMAC and survivin or other IAPs. It is also unknown how UC-112 suppresses the expression of survivin and other IAPs. However, it appears that UC-112 may reduce their stability via proteasome-dependent manners. Computational docking analysis predicts that UC-112 may bind to the BIR domain of survivin although experimental evidence is needed to prove this binding. It also remains to be determined why disrupting the interaction between SMAC and IAPs by UC-112 would lead to degradation of IAPs. UC-112 likely has other unknown activities that would facilitate the degradation of survivin and other IAPs, such as changing the conformation of these proteins. Many questions remain to be answered in order to further develop UC-112 as a SMAC mimetics to inhibit survivin and/or other IAPs.

In a follow-up structure-activity relationship study, analogs of UC-112 were synthesized and analyzed [165]. One of the analogs, 4g, has an iso-propyl group substituted on the C-linker of UC-112 (**Table 4**) with a 4-fold increase in activity in growth inhibition of cancer cell lines and increased selectivity to survivin as compared to UC-112. 4g also proved effective in inhibiting the A375 melanoma xenograft in vivo similar as the parent compound UC-112. The successes of these studies certainly warrant further mechanistic investigation of SMAC mimetics as survivin inhibitor and for potential development. SMAC mimetics represent an approach that inhibit survivin interaction with other proteins by binding to survivin and, thus, eliminate other effects by binding to its partners as in the case of shepherdin. However, modification and optimization of SMAC mimetics are likely needed to increase its specificity to survivin over other IAPs, which may prove to be difficult since their binding site, the BIR domain, is the consensus signature domain of IAPs.

Withanone is a herbal ligand from the plant *Withania somnifera*, which was predicted to also bind to the BIR domain of human survivin using computational docking analysis and to interfere the caspase inhibitory function of survivin [166] similar as UC-112. However, no experimental testing has been conducted on withanone to determine if it inhibits or binds to survivin although withanone has been shown previously to have mild activity in inhibiting proliferation of various cancer cell lines [167-169]. Clearly, more studies are needed to investigate if this natural product hold any promise in binding to survivin and inhibiting its interaction with SMAC.

In another study, the protein-protein interaction sites in survivin were analyzed in-silico and hotspot residues were identified and used to generate a potential pharmacophore that may interfere survivin interaction with its binding partner chromosome passenger complex (CPC) [170]. HIV protease inhibitors, particularly Indinavir, was found to match the pharmacophore. Although Indinavir was able to inhibit the survival of MDA-MB-231 breast cancer cells with an IC_{50} greater than 500 μM, it is unknown if Indinavir binds to survivin and inhibits its interaction with its binding partners such as those involved in the formation of the CPC. Interestingly, Indinavir decreased the XIAP protein level and increased caspase 3 cleavage. It remains to be determined if this effect was via binding to and inhibiting survivin. It is noteworthy that another HIV protease inhibitor, Nelfinavir, has previously been shown to potentiate Imatinib cytotoxicity in meningioma and inhibited survivin expression [171] while Indinavir did not affect survivin expression. Thus, it is unclear if these HIV protease inhibitors actually bind to and inhibiting survivin interaction with its ligand proteins. Clearly, more studies remain to be conducted on these HIV protease inhibitors to determine if they bind to or act on survivin and can be repurposed as potential new anti-cancer therapeutics by binding to and inhibit survivin. However, because of its high IC50 at 500 μM, Indinavir will unlikely be repurposed as a survivin-targeting anticancer drug.

Due to lack of known enzymatic activities and, thus, lack of high throughput assays for survivin, Abbot Laboratories conducted an NMR- and affinity-based screening of their libraries for compounds binding to survivin and

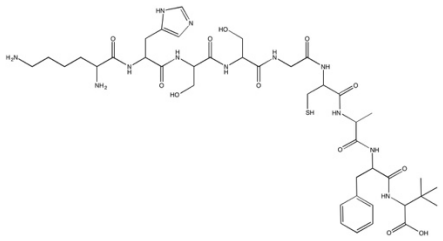
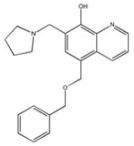
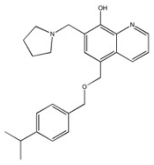
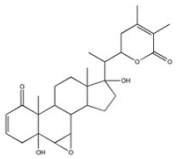
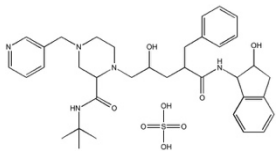
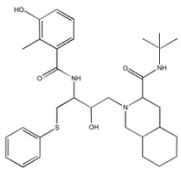
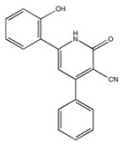
identified Abbot 8 that binds to a pocket in survivin, with a Kd of 75 μM, that may affect protein-protein interaction [172]. Via structure-activity analysis, modification of Abbot 8 increased its affinity in binding to survivin. However, Abbot 8 and its analogs were not tested for their activity in suppressing cancer cells or in inhibiting survivin interaction with its binding partners in this initial report.

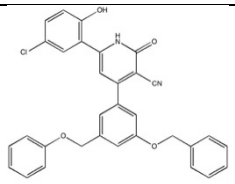
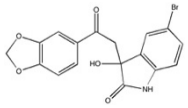
Using Abbot 8 as a lead compound targeting a site that may affect protein-protein interaction, three compounds (LLP3, LLP6, and LLP9) were synthesized and tested as survivin modulators [173]. While LLP6 has no effect on cancer cells, LLP3 and LLP9 were able to delay mitosis and inhibit proliferation of HUVEC and PC3 cells. When tested against two isogenic glioma cell lines, LLP3 has an IC_{50} of 13.6-38.1 μM. LLP3 was shown to be able to disrupt the interaction between survivin and its binding partner Ran [174], which may be responsible for the anticancer activity of LLP3. Thus, Abbot 8 and the LLP derivatives may work by inhibiting survivin interaction with its binding partners. Additional studies are clearly needed to investigate the fate of survivin following the binding of LLP compounds and survivin release from its binding partners in the CPC. Similar to LLP3, S12 was identified as a survivin-targeting molecule capable of binding to a cavity in survivin that may induce conformational changes in the protein structure that disrupt normal functions of survivin [175]. S12 treatment alters microtubule dynamics in cancer cells, resulting in disruption of spindle formation, misalignment of chromosomes during metaphase, and G2/M cell cycle arrest. S12 also inhibits cancer cell proliferation in vitro and suppresses growth of pancreatic xenograft tumors in a dose dependent manner without

effecting the overall expression of survivin. In an additional study, S12 also inhibited the proliferation and growth of sonic hedgehog driven medulloblastoma cancer cells by inducing cell cycle arrest and apoptosis [176]. The detailed mechanism of action of S12 and whether it affects survivin interaction with its binding partners is awaiting to be investigated.

In summary, the strategy targeting survivin interaction with its binding partners helps circumvent the issues encountered in targeting upstream regulators of survivin expression. This strategy may result in true survivin inhibitors that bind directly to survivin protein. However, limitations also exist with this approach. First, inhibitors such as shepherdin do not bind to and inhibit survivin. Instead, it binds to and inhibits survivin's binding partner Hsp90, which also regulates the stability of other proteins in addition to survivin. Thus, inhibitors such as shepherdin are not specific to survivin and may represent Hsp90 inhibitors. Secondly, inhibitors such as UC-112 bind to the BIR domain, which is a consensus signature domain of IAPs. Thus, it may prove to be challenging to generate a true survivin selective inhibitor that does not affect other IAPs. Thirdly, it is currently unknown what other protein partners of survivin would be affected by the inhibitors such as LLP3 designed to inhibit survivin interaction with other protein partners. Finally, the data on the mechanism of action of this category of inhibitors, especially LLP compounds and S12, are limited for detailed evaluation. More studies are needed to determine the value of the strategy in targeting survivin interaction with its binding partners.

Table 4. **Inhibitors targeting survivin interaction with its ligand proteins.**

Name	Structure	Remark	Ref.
Shepherdin		In-vitro IC50 of 25-75 μM. In-vivo xenograft model of prostate cancer line	[162]
UC-112		In-vitro IC50 of 2.1 (0.7-3.4) μM. In-vivo xenograft models of melanoma	[164]
4g		In-vitro IC50 of 0.5 μM. In-vivo model of melanoma cell line	[164], [165]
Withanone		In-silico prediction only. No experimental data	[166], [167-169]
Indinavir		In-vitro IC50 of >500 μM (MDA-MB-231)	[170]
Nelfinavir		Inhibits survivin expression. In-vivo xenograft of meningioma.	[171]
Abbot 8		In-vitro Kd of 75 μM	[172]

LLP3		In-vitro IC50 of 13.6-38.1 μM. In-vivo xenograft model of glioblastoma multiforme	[173], [174]
S12		In-vitro IC50 of 6-8 nM (colorectal cancer cells) In-vivo xenograft models of pancreatic and medulloblastoma	[175], [176]

1.5.1.3 Survivin Vaccine and Immunotherapy

The fact that survivin is over-expressed in many cancers but not expressed in most adult normal tissues has led many to believe that it may have potential to serve as a cancer vaccine for immunotherapy. Although this concept is relatively new, there have been therapeutics fast tracked to clinical trials (**Table 6**). In one study, it was found that an 8-amino acid peptide survivin-2B80-88 (A^{80}YACNTSTL^{88}), derived from survivin 2B, a splicing variant of survivin, binds to HLA-A24 and is recognized by $CD8^+$ cytotoxic T lymphocytes [177]. Cytotoxic T lymphocytes induced by survivin-2B80–88 ex vivo are capable of recognizing and acting on cancer cells expressing HLA- A24 and presenting endogenously processed survivin-2B peptide in tumor cells. Also, HLA-$A24^-$ restricted cytotoxic T lymphocytes from peripheral blood mononuclear cells of HLA-$A24^+$ breast, colorectal, and gastric cancer patients can be induced by the survivin-2B80–88 peptide and they effectively cause cytotoxicity against HLA-$A24^+$ but not HLA-$A24^-$ lung adenoma A549 cell line [178]. These data formed the basis for a phase I clinical trial using the survivin-2B80–88 peptide in patient with advanced or recurrent breast and colorectal cancers [179, 180]. Although survivin-2B80-88 was well tolerated, it unfortunately did not elicit significant clinical response alone or in combination with incomplete Fruend's adjuvant in these patients.

In a similar study, another survivin peptide mimetic SVN53-67/M57-KLH or SurVaxM that can be presented by MHC class I to induce $CD8^+$ cytotoxic T

lymphocytes [181]. Immunization of C57BL/6 mice with this peptide resulted in rejection of orthotopic GL261 glioma xenografts. SVN53-67/M57 also stimulated cytotoxic T lymphocyte responses against human tumor cells of several different MHC class I haplotypes ex vivo. Currently, two ongoing clinical trials for newly diagnosed glioblastomas and multiple myelomas are actively recruiting participants to test the efficacy of SVN53-67/M57.

Cancer immunotherapies targeting survivin certainly offers an exciting approach for treating cancers. The ability to direct the immune system to recognize and kill cancer cells is revolutionizing cancer therapy. While survivin peptides and mimetics have yet to demonstrate significant clinical efficacy in immunotherapy, it is promising that they appear to be well tolerated and do not produce apparent toxicities. Also, it may not induce aberrant immune reactions as some other immunotherapeutics. Clearly, this approach is exciting as it may be a way to produce a robust immune response specifically against cancer cells that overexpress survivin.

Table 5. **Immunotherapies targeting survivin.**

Names	Sequence	Remark	Reference
Sur-2B80-88	AYACNTSTL	Lack of clinical response in phase I trial	[177], [178], [179, 180]
SurVaxM	DLAQCFFMFKELEGW	In-vivo glioma xenograft model. Clinical trials ongoing	[181]

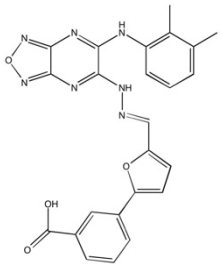
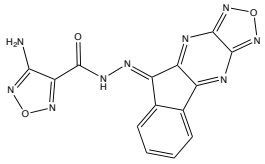
1.5.1.4 Inhibitors Targeting Survivin Homo-Dimerization

Because survivin has no known enzymatic activities but works as a homo-dimer, targeting its homo-dimerization for identifying and developing survivin inhibitors has recently been attempted [61]. This approach not only helps circumvent some of the issues encountered in targeting upstream regulators of survivin expression and result in true survivin inhibitors that bind directly to survivin proteins, it may also help eliminate survivin protein, resulting in spontaneous apoptosis due to survivin elimination. Because the homo-dimerization interface of survivin is unique, the inhibitor to this site is likely to be selective to survivin over other IAPs.

As discussed above, the buried accessible area in the homo-dimeric interface of a monomeric survivin is 550 $Å^2$ and occupies only 6% of the total accessible area of a monomer (9,044 $Å^2$) compared with ~20% in most other dimeric or oligomeric proteins. Thus, targeting the homo- dimerization interface to disrupt survivin homo-dimerization would be feasible. Furthermore, disrupting survivin homo-dimerization will lead to exposure of the hydrophobic dimeric interface, which would target the protein to proteasome for degradation. In a recent in-silico screening study targeting the critical dimerization residues following detailed computational analysis of the dimerization interface, a hit molecule, LQZ-7, was identified [61] (**Table 5**). LQZ-7 not only was able to specifically disrupt survivin dimerization and inhibit cancer cell survival, it was also able to induce survivin degradation in a proteasome-dependent manner. However, it had no effect on survivin transcription or synthesis.

Further analysis of LQZ-7 analogs led to identification of superior compounds including LQZ-7F that can more effectively disrupt survivin dimerization, cause proteasome-dependent survivin degradation, and inhibit cancer cell survival. The IC_{50} of LQZ-7F against a panel of human cancer cell lines ranged from 0.4-4.4 μM and it induced spontaneous apoptosis in these cancer cells. Using pull-down assay with immobilized LQZ-7F and purified survivin, it was found that LQZ-7 can directly bind to survivin, possibly to the interface for homo-dimerization. LQZ-7F also showed promising in-vivo activity in mice. It effectively inhibited the growth of PC3 xenograft tumors and reduced survivin protein in the xenograft tumors as expected. LQZ-7F was also well tolerated without affecting the body weight of the mice [61]. Future studies of LQZ-7F and other LQZ-7 analogs as well as further optimization may prove fruitful in targeting survivin homo- dimerization for developing novel true survivin-targeting cancer drugs.

Table 6. **Inhibitors targeting homo-dimerization of survivin.**

Name	Structure	Remark	Reference
LQZ-7		In-vitro IC50 of ~20 μM for prostate cancer cell lines	[61]
LQZ-7F		In-vitro IC50 of 2.4 (0.4-4.4) μM for multiple cancer cell lines In-vivo xenograft model of prostate cancer	[61]

Docking LQZ-7 and LQZ-7F in survivin revealed how they interact with the dimerization residues (**Figure 7A and 7B**). LQZ-7 has three important interactions with survivin via (a) H-bond between an aniline NH group of LQZ-7 and Glu^{94} of survivin; (b) interaction between the substituted aniline in LQZ-7 and Phe^{93}, Phe^{101}, and Leu^{98} in the hydrophobic pocket of survivin via π-π stacking and hydrophobic interaction; and (c) H-bond between the carboxylic acid of LQZ- 7 and Trp^{10} of survivin. LQZ-7F has two key interactions with survivin via (a) H-bond between the primary amine group of LQZ-7F and Glu^{94} of survivin and (b) the tetracyclic furazanopyrazine ring of LQZ-7F interacts with Phe^{101}, Phe^{93}, Leu^{98}, and Trp^{10} via π-π stacking and hydrophobic interactions. These interactions may effectively inhibit survivin homo-dimerization.

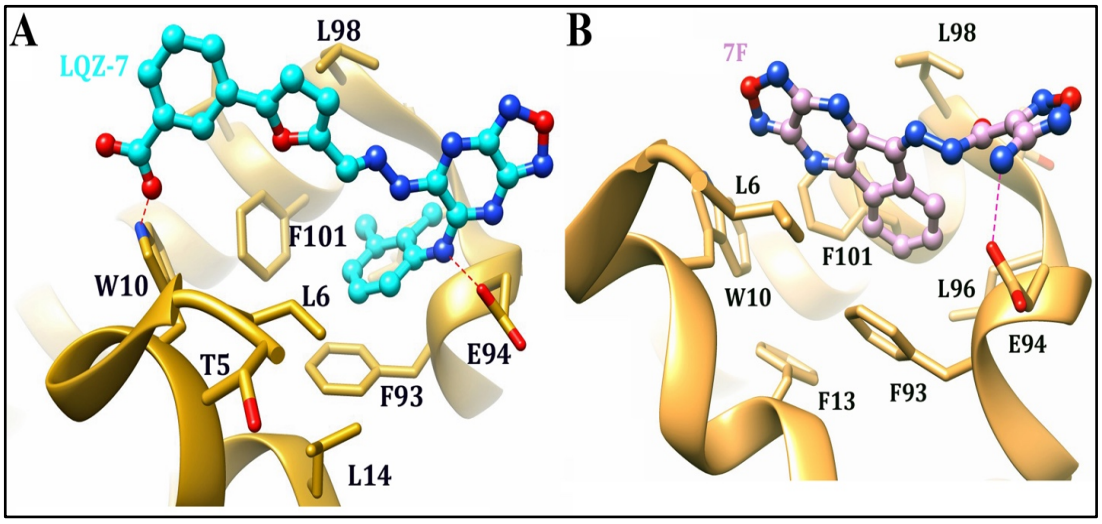


Figure 7. **Predicted binding modes of LQZ-7 and LQZ-7F in survivin**. Both LQZ-7 (A) and LQZ-7F (B) interact with important residues for survivin homo-dimerization including residues Leu^{98} and Phe^{101}. Proposed hydrogen bonding between compounds and survivin backbone are represented by dashed red lines. From *Peery and Zhang* (2017).

Based on the above pre-clinical studies, targeting survivin by disrupting its homo- dimerization may be a good strategy going forward. Although it has been shown that LQZ-7 and LQZ-7F bind to survivin, disrupt survivin homo-dimerization, and induce survivin degradation, it is yet unknown if they truly bind to the homo-dimerization interface of survivin as intended. Since this study is the only one that has shown targeting the homo-dimerization domain of survivin, caution needs to be taken when considering targeting survivin by disrupting its homo-dimerization.

More independent studies are needed to demonstrate the feasibility of this strategy in targeting survivin and possibly other homo-dimeric proteins for therapeutic discovery. It is also noteworthy that targeting homo-dimerization is more challenging than targeting survivin interaction with other proteins. It will be very perplexing to establish a high throughput assay to investigate and target homo-dimerization. In-silico screening may have to be used for such studies as demonstrated by the study discussed above.

1.6 Summary

Survivin has consistently been demonstrated to be a critical factor in tumor progression and resistance to chemotherapy. The fact that survivin is overexpressed in almost every cancer but not expressed in most adult normal tissues has positioned it as a strong tumor marker with a robust correlation to poor patient prognosis. Despite the limited response of currently available therapeutics targeting survivin in clinical trials, targeting survivin for cancer intervention remains a highly attractive option. The lack of efficacy and dose-limiting toxicities in clinical trials despite strong pre-clinical data for some inhibitors may be attributable to a deficiency in true survivin-specific inhibition. Strategies that target survivin directly by methods such as immunotherapy or direct homo-dimerization inhibitors may hold the key to overcome some of the inadequacies of previous attempts in targeting survivin moving forward. There still exists a great need to test the combination of direct survivin inhibitors with current standard chemotherapies to shed light on potential synergistic drug effects that may overcome the drug resistance paradigm in many cancers. One can certainly envision a future in which novel direct survivin inhibitors are positioned in the clinic as standalone agents or in combination with current standard of care chemotherapies.

Given the pre-clinical data, direct survivin inhibition may be most suitable as a member of a combination therapy in which the inhibition of survivin enhances the apoptotic response of tumor cells to apoptotic stimuli induced by chemotherapies. In this study, it was sought to determine if survivin is a

contributor to docetaxel resistance and the mechanism by which a survivin inhibitor may function. The data presented here lead to a new hypothesis that survivin overexpression can contribute to docetaxel resistance in prostate cancer. The goal of this work was to investigate three specific aims: (1) to implicate survivin overexpression as a molecular player in docetaxel resistant prostate cancer, (2) to perform optimization of survivin dimerization inhibitor LQZ-7 backbone and identify its mechanism of action, (3) to perform structure activity relationship analysis of LQZ-7F backbone. The long term goal of this work is to identify a novel survivin inhibitor capable of killing cancer cells and possibly synergizing with docetaxel for future translational therapies for treatment of prostate cancer.

CHAPTER 2. MATERIALS AND METHODS

Methods

Cell Culture

Prostate cancer cell lines PC-3, C4-2, Du145, 22RV-1, and LNCAP were cultured in complete growth media, RPMI-1640 (Corning, Manassas, VA) supplemented with 10% Fetal Bovine Serum (Life Technologies-Gibco, Grand Island, NY). Cells were maintained at 37^{o} C 5% CO_2 water-jacketed incubator. When confluent, cells were split by washing the flask one time with sterile 1X PBS followed by the addition of 0.05% trypsin (Lonza, Walkersville, MD) to digest the cells. After 5 minutes the trypsin was neutralized with new medium and cells were counted and seeded for desired experiment [182]. Low passage cells were maintained by splitting every 3 days and low passage cells were frozen using freezing media (50% RPMI-1640, 40% FBS, 10% DMSO) and stored in liquid nitrogen. All media and cell lines used are summarized in **Table 7**.

Stepwise Cell Selection Docetaxel Resistant Cells

The stepwise cell selection for the generation of docetaxel resistance cell lines began at a concentration 0.1 nM docetaxel for both Du145 and C4-2 which is well under the IC_{50} of these cell lines and was performed as previously described [183]. Briefly, for the selection process the desired concentration was added to the flask of cells and sensitive cells died off while resistant cells continued to proliferate. After the addition of docetaxel to the cells, surviving cells were allowed to recover to confluency before being split. Each concentration

stage was performed three times before increasing the docetaxel concentration. The selection process took roughly two weeks at each concentration. When cells were deemed ready, the concentration of docetaxel was approximately doubled and the same process was repeated. This process was stopped when the Du145 cells reached 100 nM and C4-2 cells reached 70 nM. At this point the cells are maintained in the flask with these concentrations added freshly every 48 hours. The resistance to docetaxel was confirmed by methylene blue assay.

Compounds

All compounds were kindly synthesized by Dr. Mingji Dai's Lab at Purdue University in West Lafayette, Indiana. Compounds for *in-vitro* cell-based studies were all dissolved in 100% DMSO to make a stock solution of 10 mM that was aliquoted into small aliquots for use. Compounds were stored in solution in -20^{o} C freezer for no more than a few months and freeze thaw cycles were avoided as much as possible. Compound powder was also stored with bottle sealed in parafilm at -20^{o} C for long term storage.

Western Blot Analysis

Cells were harvested for western blot analysis by washing with 1X cold PBS two times followed by scraping with a rubber policeman as previously described [184]. Cell pellets were collected by centrifugation at 500g for 5 minutes. Pellets were either stored at -80 or lysed immediately for analysis. Lysis was performed by addition of tnn buffer (50mM Tris-HCl (pH 7.4), 150 mM NaCl,

20 mM EDTA (pH 8.0), 50 mM NaF, 0.5% NP-40, 1mM Na3VO4) with the addition of 20 μl 10% SDS, 10 μl 0.2M PMSF, and 140 μl 1M DTT to every 2 mL buffer right before use. Pellets were stored on ice for 30 minutes with vortexing of each tube every 10 minutes. Cell lysates were then sonicated on ice at 40% amplification. The cell lysates were collected by centrifugation at 13,000 RPM for 10 minutes at 4° C. The supernatant was collected and aliquoted to a fresh microcentrifuge tube. Protein concentrations were calculated using Bradford Standard Assay [185].

The desired amount of protein was loaded on to SDS-PAGE gel using 2X SDS loading buffer (100 mM Tris-Cl, pH 6.8, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, 200 mM β-mercaptoethanol). Gels were run at a constant 20mA per gel for 1.5 to 2 hours. Gels were transferred to PVDF membranes overnight at constant 35 Volts at 4° C [186]. The next day the membranes were blocked in 5% milk in TBST (50 mM Tris, 150mM NaCl, pH 7.4, 0.05% Tween) for 2 hours at room temperature. The membrane was then probed with the primary antibody based on manufacturer's recommendations, most commonly 2 hours at room temperature. Blots were then washed three times for 15 minutes each using 5% milk in TBST. Secondary antibody was probed for 1 hour at room temperature based on manufacturer's recommendations. Membranes were once again washed for three times for 15 minutes each. Protein bands were detected by the addition of ECL western blot detection reagent (GE Healthcare, Chicago, IL) and X-ray film exposure [187]. The antibodies used for these experiments are summarized in **Table 8.**

Methylene Blue Assay

The assay was performed similarly to as previously described[188]. Briefly, prostate cancer cells were seeded 2000-3500 per well (cell line dependent) and treated with drugs for 72 hours. All compound dilutions were performed in cell media containing a set amount of vehicle. After 72 hours, media was then removed, and cells were fixed with methanol (Fisher Scientific) for 30 minutes and subsequently stained with 100 µL of 1% methylene blue (Sigma) (diluted in 10 mM borate buffer) for 1 hour. The cells were then washed 3 times with 10 mM borate buffer and then allowed to air dry for 30 minutes. 100 µL of 100% ethanol:0.1 M HCl (1:1) was added to each well to dissolve the methylene blue stain and absorbance was measured via spectrometry at 650 nM using a 96 well-plate reader (Walther Hall Fifth Floor Common Area). After normalization to DMSO controls, the percent viabilities were graphed and IC_{50} concentrations were determined using Graph Pad Prism software. DMSO control did not show a difference from media alone cells. Common buffers and materials for these experiments are summarized in **Table 9** and **Table 10**.

Survivin Overexpression

The C4-2 cell line was selected for survivin overexpression as it has the hallmarks and characteristics that most resemble prostate cancer characteristics seen in patients (express PSA and AR protein as well as generally form osteoblastic lesions when metastasis occurs). Before performing the stable transfection, a kill curve with the chosen selection antibiotic geneticin (G418) was

performed to determine the appropriate amount of antibiotic required to kill the majority of cells but not every cell. For C4-2 cells it was determined that 800 μg/mL was the appropriate concentration to perform the selection for the survivin plasmid.

For the stable transfection of pcDNA3.1 HA-tagged survivin and pcDNA3.1 empty vector plasmid a 10 cm dish format was utilized [189]. For each plasmid, 20 x 10^5 cells were transfected with 10 ug of either vector or survivin plasmid DNA. This was done by adding 10 ug of DNA to 700 μl of serum free media in a microcentrifuge tube. In another tube, 30 μl of metafectene pro (Biontex, Munchen, Germany) was added to 700 μl serum free media. These two tubes were combined carefully, gently mixed and stored for 20 minutes at room temperature. 9 mL of fresh media with serum was then added to each dish followed by addition of the DNA and transfection reagent mixture. The transfection was for 48 hours before each dish was split in a 1 to 10 ratio in to fresh new 10 cm dishes. The following day, G418 was added to the media and the selection for positive clones was performed. Every 72 hours fresh G418 and media were added to each dish until colonies able to be seen by the naked eye were visible. At such time, the colonies were picked using a p200 μl tip and 100 μL of trypsin to help detach the colony. Each colony was carefully transferred to a 48 well dish followed by addition of 900 μl of media containing G418. When each well reached confluency it was split by trypsinization and expanded to 2 new wells per clone in a 6 well plate. After confluency was reached at this stage one well was used for further growing up the clone in a t75 flask that would be used

to eventually confirm expression and other experiments. The other well was frozen down for a stock of each clone.

SiRNA Transfection

For the transient survivin siRNA transfection a 6 well plate format was utilized with 2 x 10^5 cells per well. A total of 4 wells was seeded in the 6 well plate, 2 wells for scramble control siRNA and 2 wells for the survivin siRNA [190]. The cells were cultured for 24 hours before the transfection was performed. For the transfection protocol, 200 μl of Opti-MEM Medium (Life Technologies-Gibco, Grand Island, NY) was added to two different microcentrifuge tubes. To one tube, 15 μl of survivin siRNA (10 μM stock) was added to one tube and 3 μl of scramble control siRNA (50 μM stock) to the other tube. In two more additional tubes, 200 μl Opti-MEM Medium was combined with 20 μl Lipo-RNAi Max transfection reagent (Invitrogen, Carlsbad, CA). The tubes containing siRNA and Lipo-RNAi max were combined, mixed gently and left at room temperature for 20 minutes. Before addition of the siRNA to cells, the media in each well was changed to fresh complete RPMI-1640 10% FBS media (1 mL). The siRNA/transfection reagent mixture was then added to each well at 200 μl/well. The transfection was performed for 48 hours before one well was split and used for methylene blue assay and the other well was harvested for western blot analysis.

Survivin Mammalian Two Hybrid

The mammalian two hybrid assay was performed utilizing the ClonTech Matchmaker Mammalian Two Hybrid Assay kit [191]. Briefly, the coding region (~400bp) of survivin was cloned in to two different plasmids, the pM plasmid containing the GAL4-DNA Binding Domain and the pVP16 plasmid containing an activation domain. For this the survivin plasmid was used as a template to do PCR to amplify the survivin coding region with the following primers:

SurF: 5’
AAGAATTCGGCGGCGGCGGCGGCGATGGGTGCCCCGACGTTGCCC 3’
SurR: 5’ AACTCGAGTCAATCCATGGCAGCCAGCTGCTC 3’

Once the PCR was complete, the survivin PCR fragment was inserted into the AT easy vector (Promega, Madison, WI). The PCR fragment was then removed from the plasmid with EcoR1, blunting, and Xho1 digestion. Then each plasmid, pM and pVP16 was digested with BamH1, blunted, and Sal1. Finally, a ligation was performed between the survivin PCR fragment and both the pM and pVP16 plasmids. Correct orientation and sequence was validated by sequencing using forward and reverse primers to survivin.

These two plasmids (0.45 μg/well) along with the pGSEAP reporter plasmid (0.09 μg/well) and firefly luciferase plasmid (30 ng/well) were co-transfected in to 1 x 10^5 Du145 cells per well in a 12 well plate [192]. Each condition was tested in triplicate. For experiments utilizing LQZ-7-3 the cells were

seeded then 24 hours later the transfection was performed. 48 hours later the media was changed, and cells were treated with DMSO or LQZ-7-3 for 24 hours. SEAP was detected utilizing the Takara ClonTech SEAP Great Escape chemiluminescence assay kit 2.0 and cells were lysed, and luciferase was measured to control for transfection efficiency. For statistical analysis RLU was normalized to luciferase value.

Luciferase Assay

In conjunction with the mammalian two hybrid assay, 30 ng firefly luciferase was transfected in to each well for a transfection efficiency control [193]. For this assay the firefly substrate was first thawed in the dark. Media from each well was collected for the two-hybrid assay, and cells were washed with 1X sterile PBS. 300 µL of Passive Lysis Buffer from the Dual Luciferase Assay Kit (Promega, Madison, WI) was added to each well. The plate was put on a rocker at room temperature and shaken at high speed to lyse the cells for at least 20 minutes. The plate was then viewed under the microscope to ensure the cells had lifted off the plate and lysed. The cells were transferred to fresh microcentrifuge tubes and collected by centrifugation for 5 minutes at 1560 RPM. Each tube was placed on a dry ice ethanol bath for 2 minutes. The tubes were then transferred to 37 degree water bath for 2 minutes. The freeze/thaw process was repeated for a total of 3 cycles. The samples were then spun at 12,000 RPM for 2 minutes. The supernatant was transferred to a fresh tube and used to perform the Luciferase Assay. For the assay 5 µL of sample and 25 µL of Firefly

Luciferase were added to a 12x75 mm Brand glass tube. The tube was shaken 3-5 times and then loaded into the luciferase machine. The value was recorded in Relative Light Units and used to normalize the two hybrid data.

IAP Family Member Degradation Assay

For this experiment C4-2 or PC-3 cells were plated in a 10 cm dish at 1 x 10^6 cells per dish. 24 hours later the media was changed, and cells were treated with DMSO or LQZ-7-3 10 μM. After 48 hours the cells were harvested, washed in PBS, and IAP family members level were evaluated using specific antibodies and Western Blotting. The antibodies were probed using the specification provided by the Cell Signaling IAP family antibody kit (Danvers, Ma). Stripping the blot and re-probing similarly sized proteins was avoided for fear of losing protein on the membrane.

Survivin Cycloheximide Half-Life Assay

The effect of LQZ-7-3 on survivin half-life was performed as previously described [194]. Briefly, PC-3 or C4-2 cells were plated in a 10cm dish at 8 x 10^5 cells and cultured for 24 hours. The dishes were then pretreated with 10 μmol/L cycloheximide for 1 hour followed by incubation with or without LQZ-7-3 10 μM for different times (0, 30 minutes, 60 minutes, 1 hour, 2 hours, 4 hours, and 6 hours). The cells were then harvested for Western Blot analysis for survivin.

Apoptosis Assay

The apoptosis assay was performed as detailed in the Annexin V-FITC kit from BioVision (Milpitas, CA) [195]. Briefly, apoptosis was induced by treatment with LQZ-7-3 at 3 μM or LQZ-7F-1 200 nM for 24-48 hours. Cells were trypsinized and collected by centrifugation at 1500 RPM. Per manufacturer directions, the cell pellet was washed one time with 2 mL of standard cell growth media. Then, cells were resuspended in 500 ul of 1X binding buffer and 5 μL of annexin V-FITC and 5 μL of propidium iodide (50 μg/ml) was added to the cells. The cells were incubated at room temperature for 5 minutes in the dark then subjected to quantification by flow cytometry via excitation at 488 nm and 530 nM. The flow cytometry was performed in the IU Simon Cancer Center Flow Cytometry Core.

Proteasome Inhibitors Study

For the proteasome inhibitor rescue experiment, PC3 and C4-2 cells were seeded in 10-cm dishes at 8×10^5 cells/dish and cultured for 48 hours followed by replacement with fresh media containing DMSO control, 7 μmol/L MG132, or 70 nmol/L bortezomib and incubation for 2 hours. LQZ-7-3 or LQZ-7F-1 was then added to the culture to final concentrations of 10 μM and 500 nM respectively and incubated for additional 24 hours. The cells were then harvested and used for Western Blot analysis of survivin to determine if proteasome inhibitors rescue survivin induced degradation by the compounds.

PC-3 Xenograft Model

For the *in vivo* efficacy study, 3 x 10^6 PC-3 cells in media were implanted into the hind flanks of 6 week old NSG male mice. For this procedure each mouse was first weighed and given an identifying feature (typically ear punch). Then the left hind flank was shaved and sterilized with ethanol. Cells were then carefully injected in normal growth media using a sterile 1 mL insulin syringe. After the tumor volume reached approximately 100 m^3 in volume, the mice were randomized in to two groups (5 mice/group). The mice were either given 200 μL vehicle (90% corn oil/10% DMSO) or LQZ-7-3 100 mg/kg vial oral gavage every other day for a total of ten treatments. Before each treatment mouse bodyweight and tumor volume by caliper was measured. At the end of the study mice were sacrificed, and tumors were extracted for western blot analysis and H&E staining of survivin.

Statistical Analysis

All statistical analysis was performed using the program GraphPad Prism 5. Each experiment was performed independently and at least 3 replicates of each experiment was performed to ensure consistent findings. Data presented is the mean ± standard deviation unless otherwise noted. All p-values were calculated using the Prism program via student t tests or one way ANOVA followed by post-hoc test depending on the number of groups being compared.

Materials

Table 7. **Media and Cell Lines**

Cell Line	Media	FBS	Origin/Characteristics
Du145	RPMI	10%	Prostate, metastasis brain, AR (-), PSA (-)
PC-3	RPMI	10%	Prostate, metastasis bone, AR (-), PSA (-)
C4-2	RPMI	10%	Prostate, Derived from LNCAP, AR (+), PSA (+)
22Rv1	RPMI	10%	Prostate, AR (+), PSA (+)
LNCAP	RPMI	10%	Prostate, metastasis lymph node, AR (+), PSA (+)
Du145-100 Doc	RPMI with 100 nM Docetaxel	10%	Du145 cells serially treated with Docetaxel until reaching 100 nM
C4-2-70 Doc	RPMI with 70 nM Docetaxel	10%	C4-2 cells serially treated with Docetaxel until reaching 70 nM
C4-2 Vector	RPMI with 200 ug/mL G418	10%	C4-2 cells transfected with pcDNA3.1 empty vector
C4-2 HA-Survivin	RPMI with 200 ug/mL G418	10%	C4-2 cells transfected with pcDNA3.1 C-terminus HA tagged Survivin

Table 8. **Antibodies**

Name	Company	Catalog #	Dilution
Actin	Sigma	JLA-20	1:2000
Anti-Mouse Secondary	Sigma	A2554	1:3000
Anti-Rabbit Secondary	Sigma	A0545	1:3000
Anti-Rat Secondary	Sigma	A9037	1:2000
Caspase 3	Cell Signaling	9662	1:1000
CIAP 1	Cell Signaling	7065	1:1000
CIAP 2	Cell Signaling	3130	1:1000
Cleaved Caspase 3	Cell Signaling	9661	1:200
HA-Tag	Sigma	H6908	1:1000
Survivin	Cell Signaling	2808	1:1000
XIAP	Cell Signaling	2045	1:1000

Table 9. **Common Buffers and Dyes**

Buffer	Make up
PBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4
TBS	50 mM Tris, 150mM NaCl, pH 7.4
PBST	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.05% Tween
TBST	50 mM Tris, 150mM NaCl, pH 7.4, 0.05% Tween
TNN	50mM Tris-HCl (pH7.4), 150 mM NaCl, 20 mM EDTA (pH 8.0), 50 mM NaF, 0.5% NP-40, 1mM Na_3VO_4, before each use add 20 µl 10% SDS, 10 µl 0.2M PMSF, and 140 µl 1M DTT to every 2 ml buffer.
2X SDS loading	100 mM Tris-Cl, pH 6.8, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, 200 mM **β**-mercaptoethanol

Table 10. **Common Materials**

Reagents	**Company**	**Cat #**	**Location**
1-Kb Quick load ladder	New England Biolabs	N0552G	Ipswich, Ma
5-mL polystyrene round-bottom tube with cell-strainer Cap	Fisher	352235	USA
Acrylamide/bis- 37.5:1	RPI	7732-18-5	Mount Prospect, IL
Ammonium Persulfate (APS)	Fisher	7727-54-0	USA
Annexin V-FITC	BioVision	NC9338235	Milpitas, CA
Docetaxel	Apex BIO	A4394	Houston, TX
Dual-Luciferase Reporter Assay	Promega	E1960	Madison, WI
ECL Western Blot detection reagent	GE Healthcare	RPN2106	Chicago, IL
G418	TEKnova	G5005	Hollister, CA
Gel Loading Dye (6X)	New England Biolabs	B7025S	Ipswich, Ma
Hoechst	Fisher	62249	USA
Instant Milk	Walmart		USA
Lipofectamine RNAi Max	Invitrogen	13778030	Carlsbad, CA
Metafectene Pro	Biontex	T040	Germany
Methylene Blue Hydrate	Sigma	M4159	St. Louis, MO
Opti-MEM Media	Gibco	51985091	Grand Island, NY
Plasmid Prep	Invitrogen	K210014	Carlsbad, CA
Pre-stained Protein Ladder	Fisher	26616	USA
Protein Assay Kit	Thermo Fisher	23200	Waltham, MA
PureLink mRNA extraction kit	Invitrogen	12183018A	Carlsbad, CA
PVDF	Fisher	162-0184	USA
Reporter Lysis 5X buffer	Promega	E397A	Madison, WI
TEMED	Fisher	110-18-9	USA
Thiazolyl Blue Tetrazolium Bromide	Sigma	M5655	St. Louis, MO
Triton X 100	Sigma	T8787	St. Louis, MO
Trypsin	Lonza	CC-5012	Walkersville, MD
Tween20	Fisher	BP337	USA

CHAPTER 3. SURVIVIN AND DOCETAXEL RESISTANCE

3.1 Background and Rationale

Castration-resistant prostate cancer (CRPC) remains the second most common cause of cancer related mortality in men [196]. After androgen deprivation has failed and the cancer has become castration resistant chemotherapy is utilized not as a curative agent but to extend the life of these patient population. As discussed, docetaxel is the first cytotoxic agent to show modest improvements in overall survival rate in patients with CRPC [33]. Unfortunately over half of these patients do not respond to treatment and ultimately all develop resistance [197].

The mechanism mediating docetaxel resistance remains unknown. It has been postulated that tubulin mutations, overexpression of drug efflux pumps, and overexpression of Inhibitors of Apoptosis proteins may all be implicated in resistance to docetaxel chemotherapy [198]. However, survivin, an inhibitor of apoptosis family member, has been identified as a mediator of chemo-resistance due to its dual roles in cancer cell survival through inhibition of apoptosis and facilitation of cell cycle progression [199].

There have been a few limited studies that have linked survivin expression to correlation with clinically biologically aggressive and docetaxel resistant prostate cancer [200]. Similarly, downregulation of survivin expression with a nonspecific inhibitor has been shown capable of reversing taxane resistance in prostate cancer cells [201]. However, because this inhibitor is

nonspecific it likely is affecting a multitude of different targets in the cell and therefore it remains unclear if this reversal in resistance is in fact due to survivin.

In this chapter it was hypothesized that survivin overexpression contributes to docetaxel resistance in prostate cancer. The work in this aim will hopefully provide the foundational experiments necessary to begin to investigate a mechanistic role for survivin in docetaxel resistance. The long-term goal of this translational study is to develop clinically relevant direct survivin small molecule inhibitors capable of sensitizing CRPCs to standard clinical therapies such as docetaxel. The results provide evidence that survivin expression is strongly correlated to docetaxel response in prostate cancer cells, docetaxel resistant cells display greater levels of survivin than docetaxel sensitive parental cells, and knockdown or overexpression of survivin increases or decreases sensitivity to docetaxel respectively.

3.2 Results

3.2.1 Survivin Expression and Docetaxel Correlation Analysis

First, to investigate if survivin contributes to docetaxel resistance in prostate cancer, the level of survivin expression in five different prostate cancer cells lines was evaluated. The prostate cancer cell lines included four castration resistant cell lines: Du145, PC-3, C4-2, and 22Rv1, as well as one androgen dependent cell line, LNCAP. Du145 and PC-3 are cell lines derived from brain and bone metastasis respectively. 22Rv1 cell line derived from a PCa xenograft that was serially propagated in mice after castration-induced regression and

relapse of the parental, androgen-dependent CWR22 xenograft. LNCAP was derived from a lymph node metastasis and C4-2 were isolated from a LNCaP cell subcutaneous xenograft tumor in a castrated mouse. The western blot analysis for survivin expression of these five cell lines showed that Du145 had the highest level of survivin expression and LNCAP had the lowest level of survivin (**Figure 8**). Interestingly, all of the castration resistant cell lines displayed a much higher level of survivin than the androgen dependent cell line. In fact, the C4-2 cell line that was derived from LNCAP cells had a greater than two fold increase in survivin level. The survivin antibody used for this experiment is a monoclonal survivin antibody that binds to residues near cysteine 60 of survivin and detects total endogenous human survivin. It does not cross react with other anti-apoptotic proteins but is limited by the fact that it does not differentiate potential isoforms of survivin.

In order to determine if the survivin expression level in these five cell lines correlates with cytotoxic response to docetaxel, a series of methylene blue assays was performed with each cell line. Methylene blue is a basic dye that is positively charged at pH 8.5. It binds electrostatically to negatively charged groups within cells, predominately phosphate moieties of nucleic acids and some charged groups in proteins in order to quantify cell number. The methylene blue assays showed similar results as the western blot analysis with Du145 having the highest IC_{50} value at 1.3 nM and LNCAP the lowest IC_{50} at 0.5 nM (**Figure 9A**).

To determine if the level of survivin expression correlates with docetaxel cytotoxicity, a correlation analysis was performed. The relative survivin level and

docetaxel IC_{50} had a strong positive correlation as the higher the survivin level the higher the docetaxel IC_{50} (**Figure 9B)**. This was confirmed by a high R^2 or coefficient of determination of 0.902 which represent a goodness of fit test with closer to be 1 a perfect fit. These data indicate that survivin expression is correlated to the docetaxel IC_{50}.

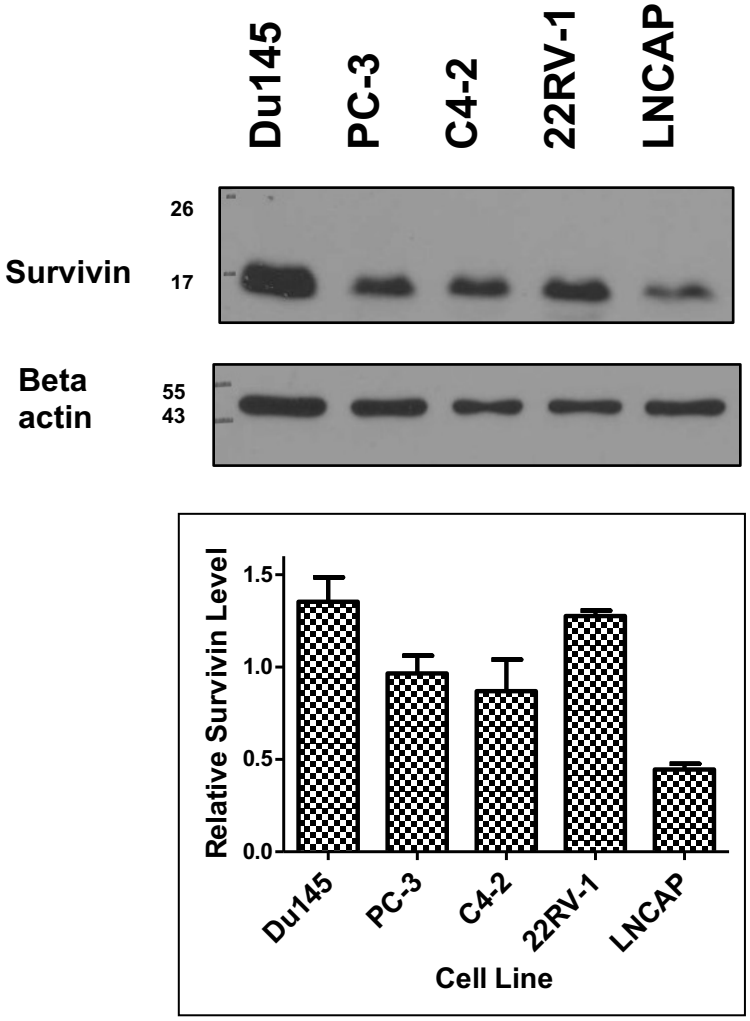
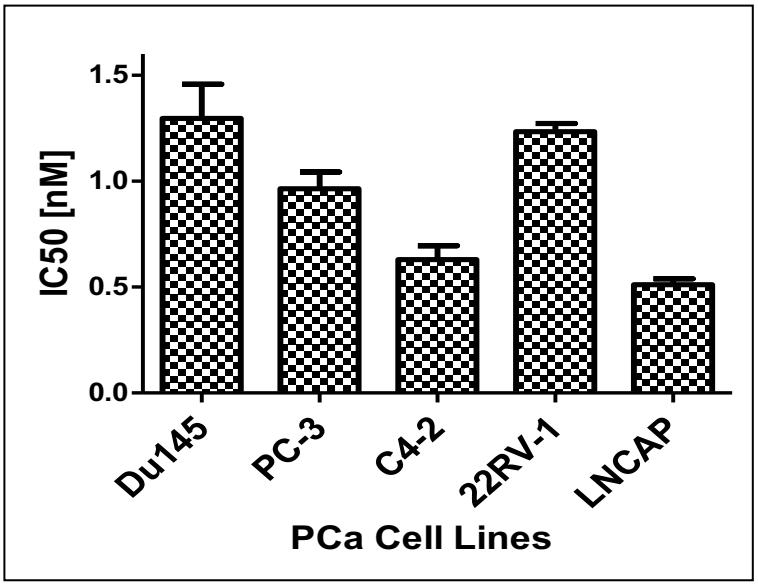


Figure 8. **Survivin protein level is higher in castration resistant cell lines than androgen dependent cell lines**. The four castration resistant cell lines have a higher level of survivin normalized to actin than androgen dependent cell lines. The error bars indicated standard deviation. n = 3 independent experiments.

A



B

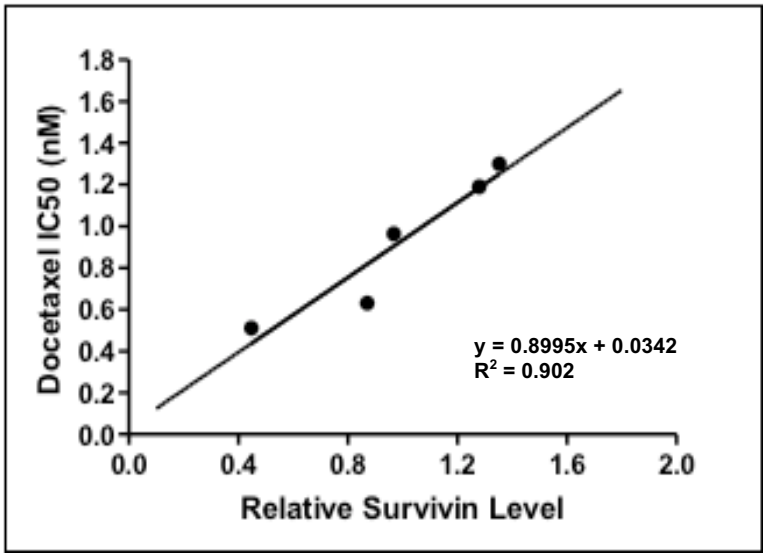


Figure 9. **Survivin protein level correlates with docetaxel IC_{50}.** (A) Average IC_{50} of methylene blue analysis of five prostate cancer cell lines. The IC_{50} was determined at 72 hour timepoint. Each concentration was tested in triplicate. Error bars represent standard deviation. (B) Correlation analysis of relative survivin level vs docetaxel IC_{50}. Strong positive $R^2 = 0.902$. n = 3 independent experiments.

3.2.2 Survivin Expression in Docetaxel Resistant Cell Lines vs. Parental Cells

In order to assess survivin potentially being a component of docetaxel resistance in prostate cancer, an in vitro model of stepwise cell selection was used in which two different prostate cancer cell lines were serially exposed to increasing concentration of docetaxel (**Figure 10**). For this selection I utilized two castration resistant cell lines, one with the highest and lowest levels of survivin, Du145 and C4-2 respectively. C4-2 cells have a number of characteristics that make them particularly desirable cell line for prostate cancer research. First, they have the nice feature of being generated from androgen dependent cell line, LNCAP. Secondly, they express prostate specific antigen and androgen receptor unlike some other prostate cancer cell lines. C4-2 cells also tend to form osteoblastic lesions when undergoing metastasis to the bone *in vivo* which correlates nicely to the osteoblastic lesions seen clinically in patients.

The Du145 cell line underwent ten total increasing escalations of docetaxel concentration from 0.1 nM to 100 nM. The C4-2 cell line underwent a total of 9 escalations of docetaxel from 0.1 nM to 70 nM. Once the cell selection process had been completed, resistance to docetaxel was confirmed by methylene blue assay. The Du145 cells were selected until 200 nM concentration, however for these studies Du145-100nM was utilized as 100 nM as it is the highest concentration of docetaxel one could expect to reach in a tumor and was consistent with the stepwise cell selection by other groups in the literature [202]. Du145-Doc cells had a significantly increased docetaxel IC_{50} of 127.8 nM as compared to parental Du145 cells IC_{50} of 1.3 nM indicating

resistance to docetaxel (**Figure 11A**). C4-2-Doc cells also had a significantly increased docetaxel IC_{50} of 84 nM as compared to parental C4-2 cells IC_{50} of 0.7 nM (**Figure 11B)**. Next, the level of survivin protein in stepwise selected resistant cells versus parental cells was determined by western blot. Du145-Doc cells showed a fivefold increase in relative survivin level compared to parental Du145 cells (**Figure 12A**). More importantly, the increased survivin protein expression was concentration dependent. C4-2 Doc cells also showed a significant four-fold increase in relative survivin level compared to parental C4-2 cells (**Figure 12B**). These data provide evidence that increased docetaxel resistance and exposure increases survivin protein level in prostate cancer cells.

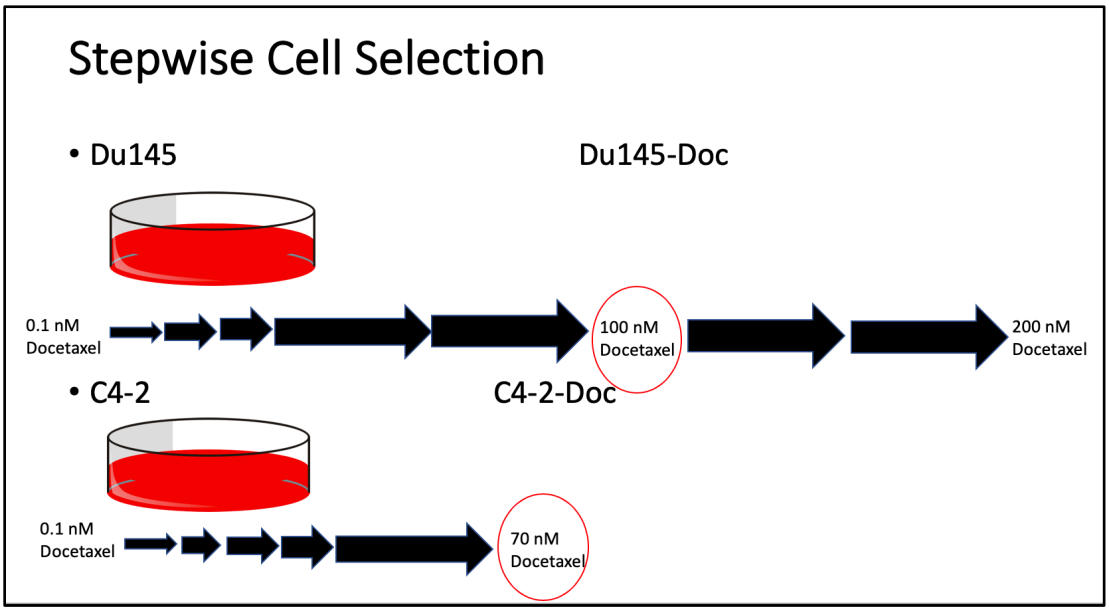
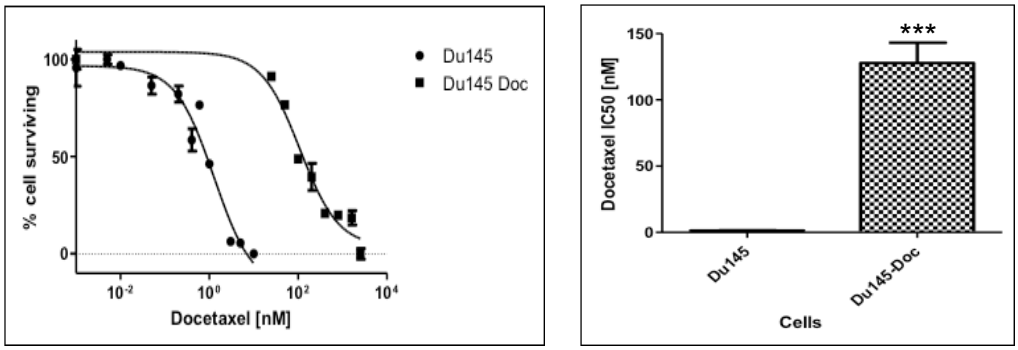


Figure 10. **Stepwise cell selection of Du145 and C4-2 cells by increasing exposure to docetaxel.** Du145-Doc cells reached a maintenance concentration of 100 nM docetaxel after 10 total steps. C4-2 Doc cells are maintained at 70 nM docetaxel after 9 total steps. Larger arrows meant to indicate a higher concentration of docetaxel used. Cells were allowed to grow to confluency a total of three times at each concentration step. Each step took roughly two weeks to complete.

A



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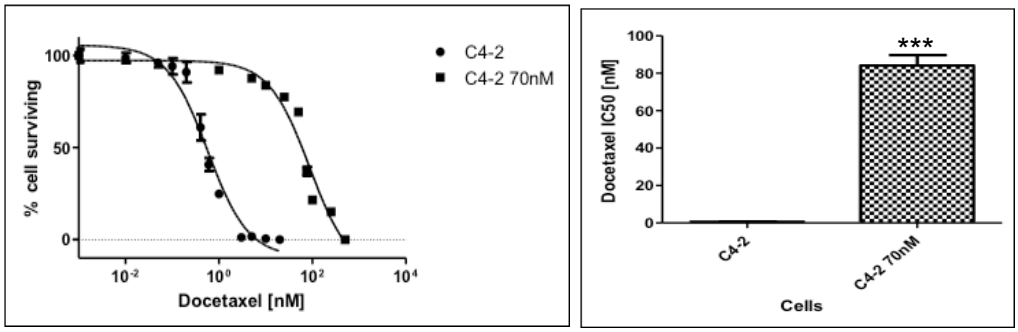


Figure 11. **Stepwise selected cells are resistant to docetaxel.** (A and B) Du145-Doc cells and C4-2 Doc cells methylene blue concentration curve is shifted to the right indicating decreased sensitivity to docetaxel and an increased IC_{50}. Each concentration was tested in triplicate and the IC_{50} was determined at 72 hours post addition of docetaxel. n = 3 independent experiments. *** = p-value <0.001. Error bars indicate standard deviation.

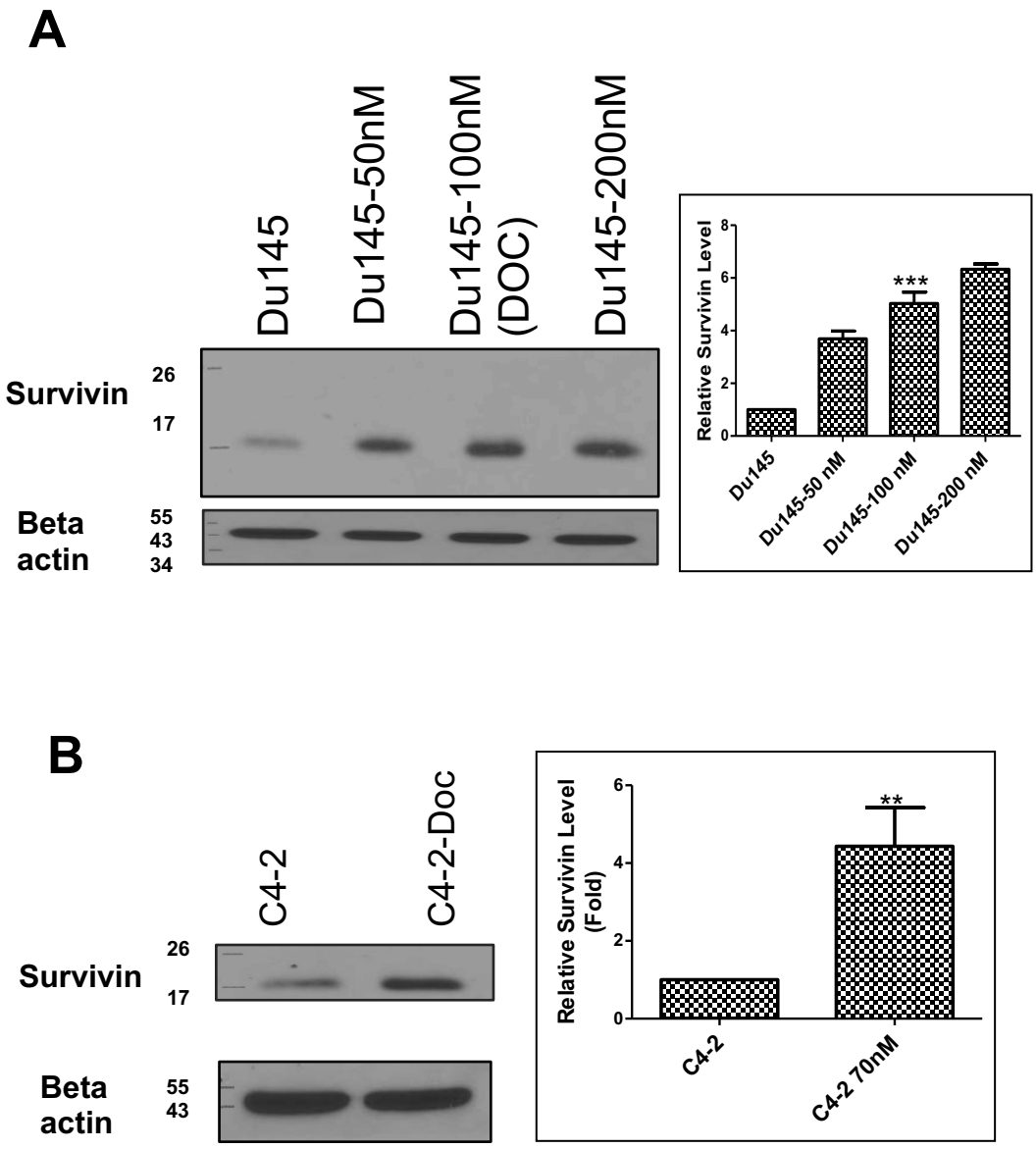


Figure 12. **Stepwise selected cells have increased survivin expression.** (A) Du145-Doc cells have five-fold increased relative survivin level. n = 3 independent experiments. *** = p-value <0.001. Error bars indicate standard deviation. (B) C4-2 Doc cells have four fold increased relative survivin level. n = 3 independent experiments. ** = p-value <0.01. Error bars indicate standard deviation.

3.2.3 Survivin Overexpression and Knockdown Effect on Docetaxel Cytotoxicity

In order to more directly determine how manipulation of survivin level may effect sensitivity to docetaxel treatment, C4-2 survivin overexpressing cells were established. Previously in our lab, a C-terminus HA-tagged survivin insert was cloned in to a pcDNA3.1 vector. This construct and control pcDNA3.1 empty vector were stably transfected in to C4-2 cells using 800 μg/mL G418 selection to establish the C4-2 survivin and C4-2 vector cell lines necessary for this set of experiments. For the following experiment utilizing these stable cells, three biological replicates were performed utilizing early passage cells that had been frozen down at -80.

To confirm the expected survivin overexpression had been stably established in these cells, western blotting was performed for survivin (**Figure 13A**). An HA-tagged antibody was also utilized as a source of secondary confirmation of overexpression plasmid. Due to the small size of survivin, the HA-tag does result in a molecular weight shift as expected in the western blot confirming overexpression. This is consistent with previous experiments performed with a HA- Tag survivin construct in mammalian cells [173]. To assess if overexpression of survivin in these C4-2 altered response to docetaxel treatment, methylene blue assays were performed. Interestingly, C4-2 survivin cells had an IC_{50} of 1.3 nM compared to 0.5 nM IC_{50} for C4-2 Vector cells (**Figure 13B**). The overexpression of survivin in these cells represented a greater than two fold increase in docetaxel required to kill fifty percent of the cells.

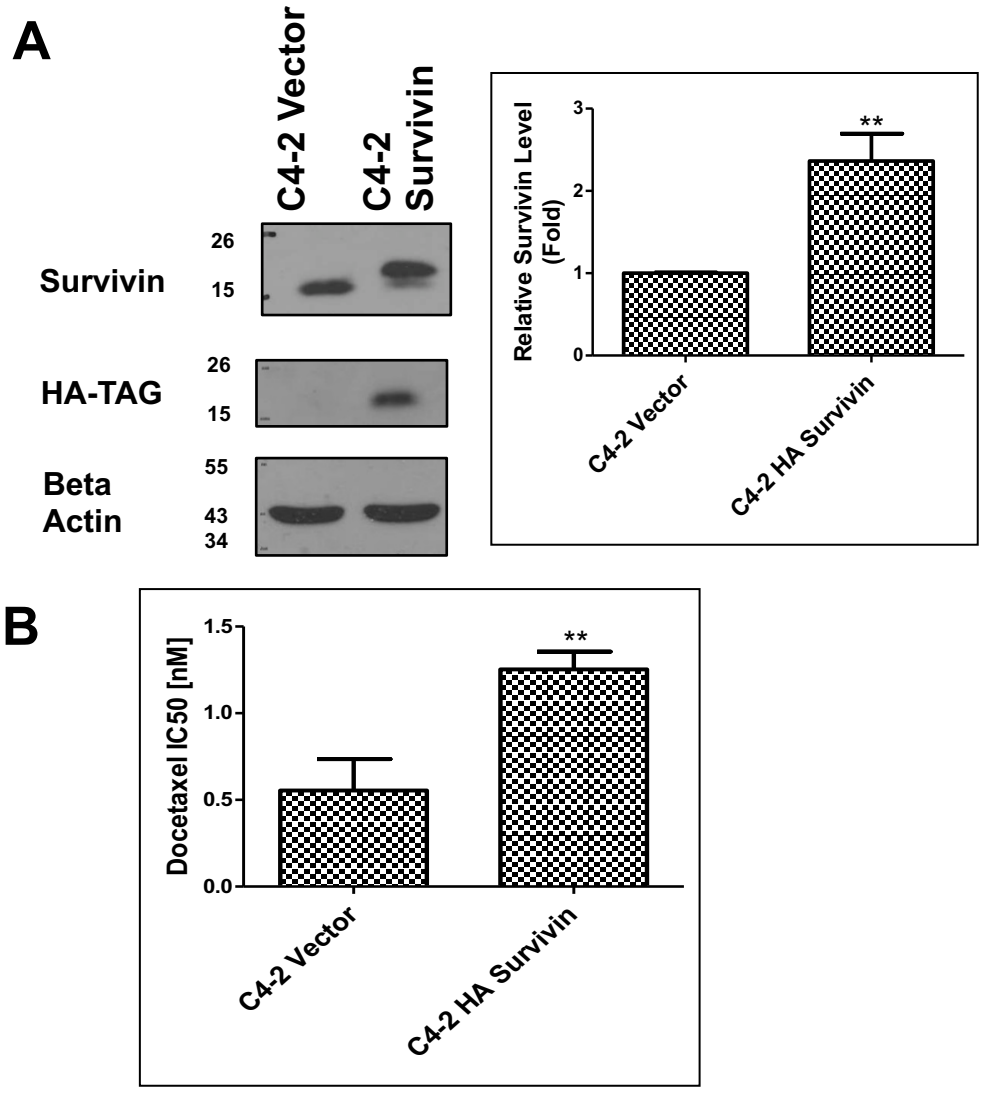
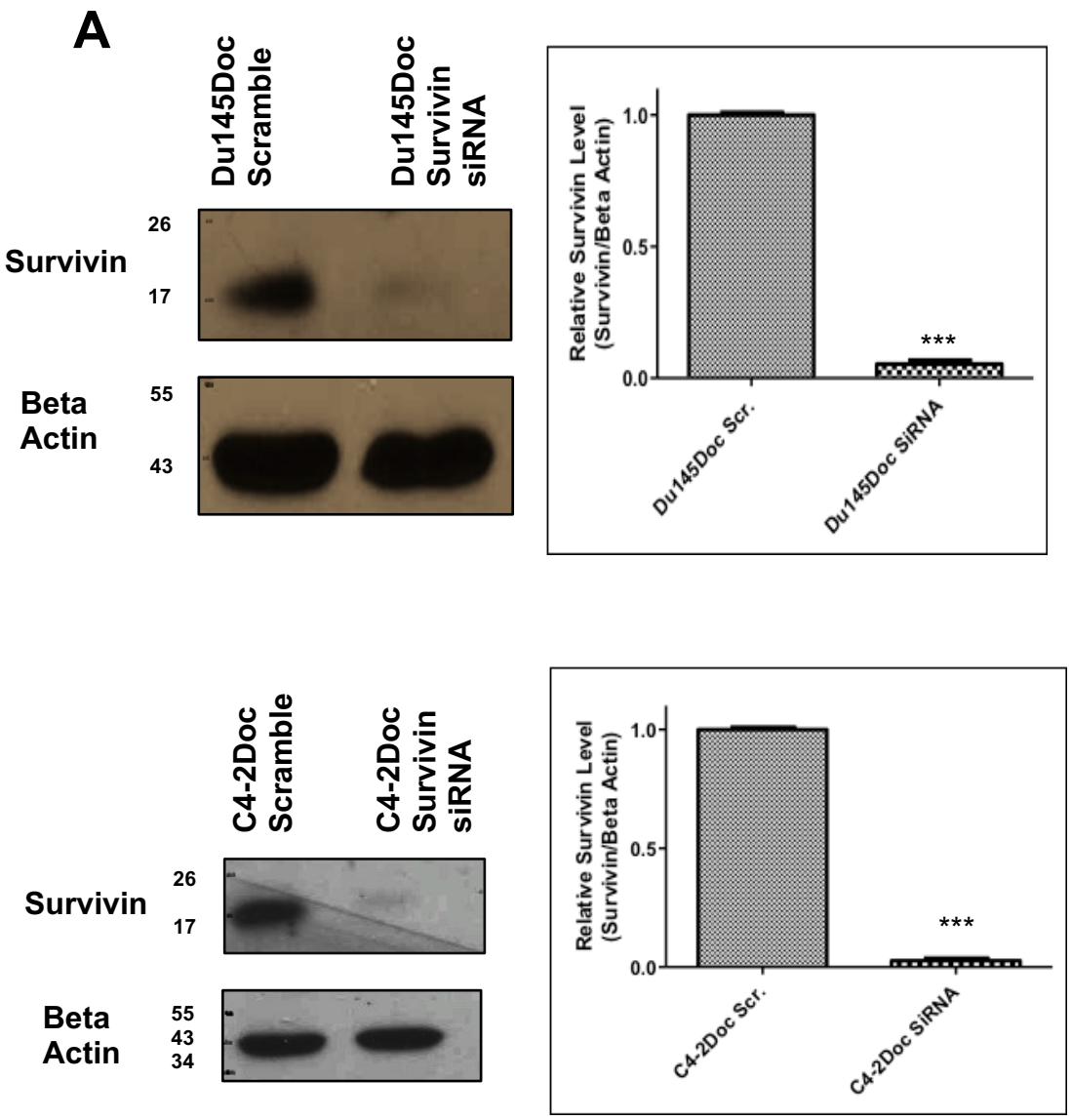


Figure 13. **Cells stably overexpressing survivin are more resistant to docetaxel.** (A) Western blot analysis of C4-2 Vector compared to C4-2 Survivin overexpression cells. Since survivin is such a small protein the small molecular weight shift caused by HA-Tag is expected. HA antibody was used to confirm stable transfection of C-terminus HA tagged survivin. n = 3 independent experiments. ** = p-value <0.01. Error bars equal standard deviation (B) Methylene blue comparison of C4-2 Vector and C4-2 survivin cells. C4-2 survivin cells have significantly higher IC_{50} value compared to vector cells. Each concentration was tested in triplicate. n = 3 independent experiments. ** = p-value <0.01. Error bars equal standard deviation.

Since our docetaxel resistant cell lines expressed a significantly higher level of survivin than our parental cell lines, it was also of particular interest to determine if transient knockdown of survivin using siRNA could result in a decreased resistance to docetaxel and lower the IC_{50} of these cells to closer to that of the parental cells. For this experiment the two docetaxel resistant cells lines were transiently transfected with 100 nM survivin siRNA, then split and plated for our standard methylene blue assays or western blot analysis. A scrambled siRNA transfection was performed in each cell line as a control.

The knockdown of survivin was first confirmed in Du145-Doc and C4-2-Doc by comparison to scramble siRNA transfected cells using western blot analysis (**Figure 14A**). Scramble siRNA is a negative control in which the nucleotide targeting sequence is randomly rearranged so that it should not target any known genes. This control is meant to serve as a control that should have similar gene expression profile of un-transfected cells. As expected, in both Du145-Doc and C4-2-Doc cells, transfection with survivin siRNA significantly reduced survivin protein level. To determine the effect of knocking down survivin in our resistant cells, a methylene blue assay was performed in comparison to scramble transfected cells. In both Du145-Doc and C4-2-Doc cells transfected with survivin siRNA, the concentration curves shifted significantly to the left, indicating a decrease in resistance to docetaxel (**Figure 14B**). Although the IC_{50} was not lowered to that of the original Du145 and C4-2 parental cells before docetaxel exposure, this may be attributed to the stepwise selection process selecting for other factors that may confer resistance in these cells. However, the

data indicate stable overexpression of survivin decreases prostate cancer cell sensitivity to docetaxel and knockdown of survivin in resistant cells lines dramatically lowers these cells resistance to docetaxel. The dramatic decreases in IC_{50} after knockdown in the resistant cells suggests once again that survivin is a major contributor to docetaxel resistance in PCa cells.


A
Du145Doc Scramble
Du145Doc Survivin siRNA
Survivin
26
17
Beta Actin
55
43
Relative Survivin Level (Survivin/Beta Actin)
1.0
0.5
0.0
***
Du145Doc Scr.
Du145Doc SiRNA
C4-2Doc Scramble
C4-2Doc Survivin siRNA
Survivin
26
17
Beta Actin
55
43
34
Relative Survivin Level (Survivin/Beta Actin)
1.0
0.5
0.0
***
C4-2Doc Scr.
C4-2Doc SiRNA

B

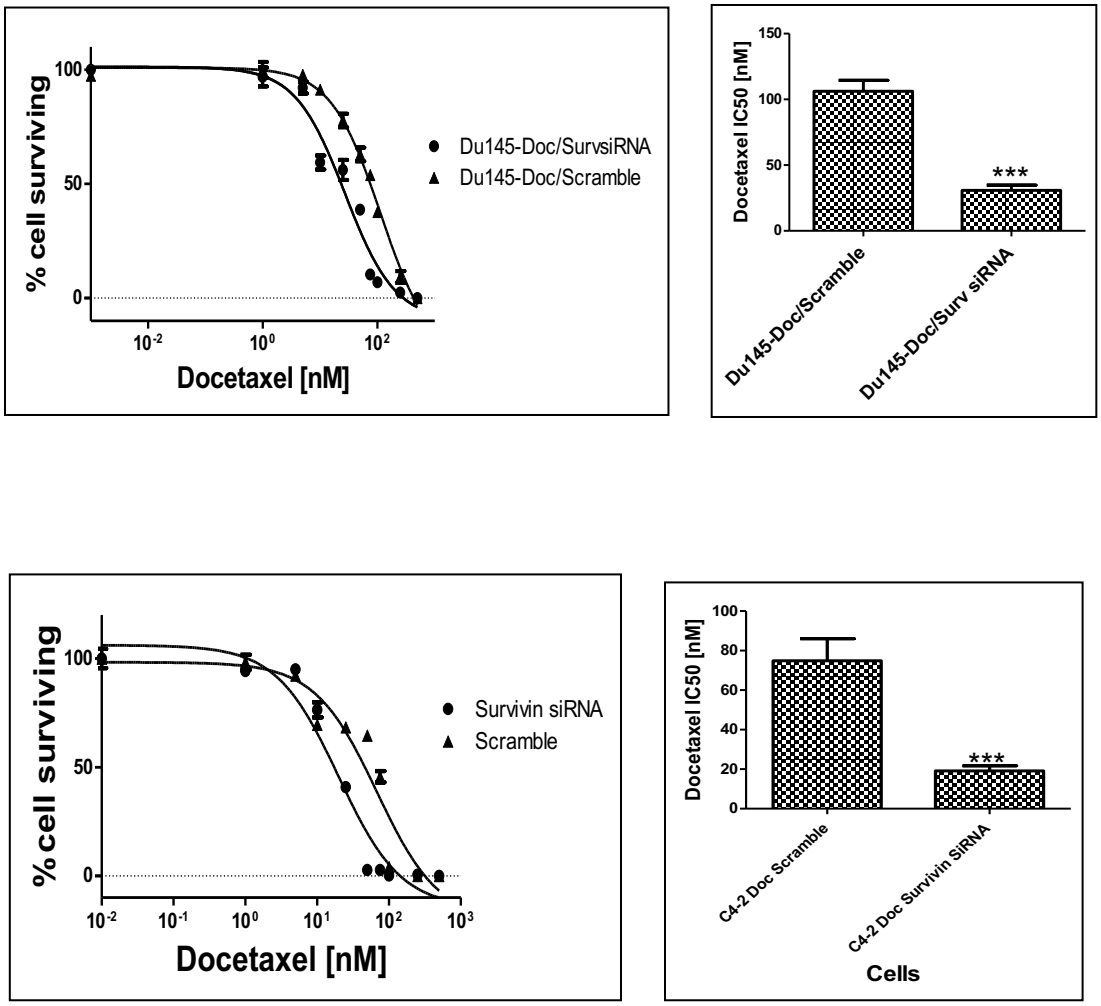
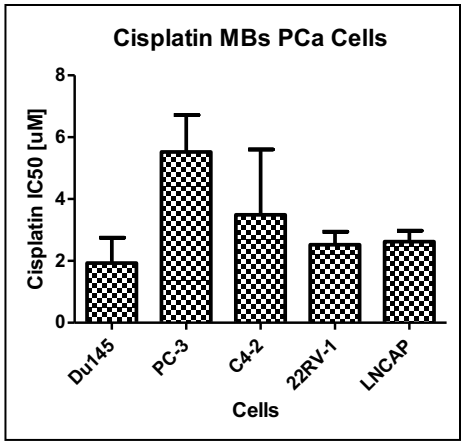


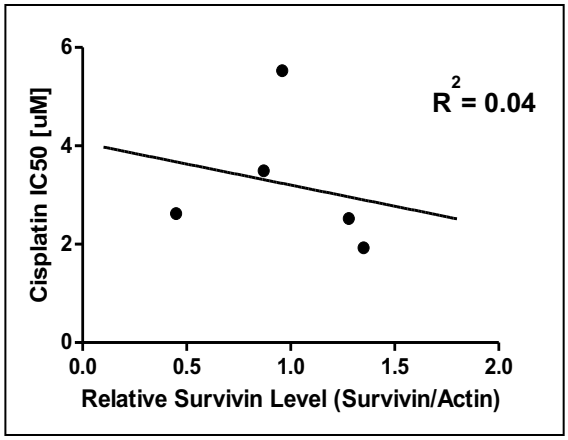
Figure 14. **Survivin knockdown by siRNA in docetaxel resistant cells decreases resistance to docetaxel treatment.** (A) Western blot analysis of scramble siRNA or survivin siRNA in resistant cell lines. n = 3 independent experiments. *** = p-value <0.001. Error bars equal standard deviation. (B) Methylene blue comparison of scramble siRNA or survivin siRNA in resistant cell lines. Survivin siRNA transfected cells have significantly lowered IC_{50} than scramble transfected cells. Each concentration was tested in triplicate. n = 3. *** = p-value <0.001. Error bars equal standard deviation.

3.2.4 Survivin Expression and Cisplatin/Doxorubicin Cytotoxicity Correlation Analysis

As noted above, survivin expression had a robust positive correlation with docetaxel cytotoxicity in the five different prostate cancer cell lines. In order to determine if other chemotherapeutics with different mechanisms of action also displayed this correlation in prostate cancer cells, methylene blue assays were performed using cisplatin and doxorubicin. Cisplatin is common chemotherapeutic for many solid cancers, like bladder cancer, that works as an alkylating agent preventing proper DNA repair and synthesis. Doxorubicin is a common chemotherapy for treatment of breast cancer that acts as a DNA intercalator and a topoisomerase II inhibitor. Interestingly, unlike docetaxel, cisplatin or doxorubicin did not show any correlation with survivin expression in prostate cancer cells with R^2 values well below 0.1 (**Figure 15**). From our data it appears that survivin expression is uniquely correlated with docetaxel cytotoxicity in our prostate cancer cell setting.

A





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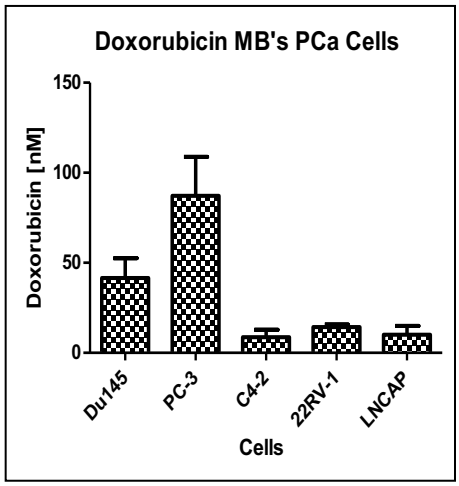


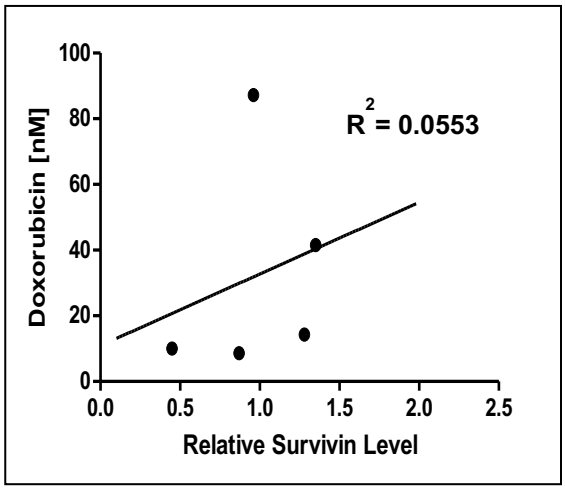


Figure 15. **Survivin expression does not correlate with cytotoxicity to cisplatin or doxorubicin in prostate cancer cells.** Correlation analysis of survivin expression in prostate cancer cells and (A) cisplatin or (B) Doxorubicin cytotoxicity. Each concentration was tested in triplicate. n = 3 independent experiments. R^2 is the coefficient of determination value representing a goodness of fit test. Error bars equal standard deviation.

3.3 Concluding Remarks

The data from this chapter serves to implicate survivin as an important molecular player in docetaxel resistance in prostate cancer. The findings above show a strong positive correlation between survivin expression and docetaxel cytotoxicity. The same was not true for other solid tumor chemotherapeutics cisplatin and doxorubicin. Stepwise docetaxel selected cells displayed significantly increased survivin expression. Cells stably overexpressing survivin had increased resistance to docetaxel. Survivin knockdown by siRNA in docetaxel resistant cells partially restored their response to docetaxel treatment. The data presented in this chapter serves to provide a foundation and rationale for exploring the molecular mechanism by which survivin may be promoting docetaxel resistance. More importantly, these data indicate survivin may be an important drug target in docetaxel resistance and its inhibition may help restore cancer cells sensitivity to drug treatment.

CHAPTER 4. LQZ-7 FLEXIBLE SCAFFOLD OPTIMIZATION

4.1 Background and Rationale

It has been previously assumed that survivin should belong to a class of 'undruggable' proteins due to its lack of enzymatic activities. As detailed above, every attempt at targeting survivin previously involved targeting the protein indirectly [131]. However, more recently our lab using a combination of computational analysis of survivin's dimerization interface and *in silico* screening, identified the first direct small molecule inhibitor of survivin protein itself which targets the residues of Leu^{98} and Phe^{101} in the dimerization interface of survivin [61]. The inhibitor identified, LQZ-7, is thought to disrupt survivin dimerization, destabilize survivin, and lead to its clearance by the cellular mechanism such as the proteasome. This study represented the identification of the first small-molecule inhibitor to target the survivin protein directly and provided a blueprint for development of more *in silico* screening based approaches to therapeutically target homodimeric proteins.

In this chapter, it was hypothesized that an optimized analogue of LQZ-7 scaffold targeting survivin will demonstrate enhanced anti-cancer activity in cancer cell lines and in a xenograft model as compared to the parental LQZ-7 compound. After chemical synthesis of five unique LQZ-7 analogs by our collaborators, the above hypothesis was tested using several cell-based assays and a PC3 *in vivo* xenograft model. The data presented in chapter identified an active analogue, LQZ-7-3, which demonstrated enhanced potency in multiple prostate cancer cell lines and suppressed xenograft tumor growth in an oral

formulation. LQZ-7-3 also lead to PC-3 and C4-2 apoptosis as measured by cleaved caspase 3 and annexin v flow cytometry staining increases as well as proteasome dependent survivin degradation. In this chapter I hypothesized that an optimized LQZ-7 inhibitor would have improved cellular accumulation and potency, leading to improved cancer cell killing. The specific aim of this chapter was to elucidate any identified analogue's mechanism of action and asses its potential as an anti-cancer agent.

4.1.1 Discovery of LQZ-7

Survivin as a non-enzymatic protein represented a particularly unique challenge in order to target the survivin protein directly. However, our lab used the knowledge that exposure of the hydrophobic core interface of a dimeric protein can cause changes in protein folding that result in weakening of the protein structure and degradation by cellular clearance mechanisms of the proteasome or autophagy [203-205]. Therefore, survivin as a homodimeric protein could possibly be targeted by screening for compounds capable of inhibiting survivin dimerization. Inhibition of survivin dimerization should then result in its clearance and prevention of its role in promoting cell proliferation and inhibiting apoptosis that cancer cells take advantage of in order to continue to grow. The discovery of LQZ-7 ended up being a two prong process: molecular dynamic simulations of the survivin dimeric interface to identify the key core critical residues for dimerization and *in silico* screening of small molecule compounds that may inhibit survivin dimerization.

Dr. Zhang and Dr. Liu took advantage of a new method they developed for identifying dimerization core units involved in homodimerization by a molecular dynamics simulation analysis of water trafficking assay [206, 207]. This analysis determined that survivin has a particularly large proportion of hydrophobic residues in the dimerization interface making it ideal candidate for an inhibitor that can disrupt dimerization and expose the hydrophobic core residues. This computational approach also identified that survivin has a singular dimerization core unit which consists of Leu^{98} and Phe^{101} from one chain and Leu^{98} and Phe^{101} from the other chain. Finally, the survivin dimerization core during the simulation had a 0.5 water/200ps exchange, which indicated very few molecules moved in and out of the core during the simulation, and is comparable to other homodimeric proteins like 14-3-3σ [61]. In total, the molecular dynamics simulation indicated that survivin has a firmly sealed dimerization core promoted by Leu^{98} and Phe^{101} that may be suitable for disruption by small molecules. In fact a previous study has indicated that mutation of Leu^{98} to alanine causes disruption of survivin dimerization [208].

The second step in the discovery of LQZ-7 involved a structure based *in silico* screening of a 200,000 small molecule compound library to identify compounds capable of targeting Leu^{98} and Phe^{101}. The program utilized for this screening was DOCK, in which the three-dimensional structure of survivin was programmed into the simulation and each compound was docked with survivin. The compounds were scored by multiple stages of energy scoring. The top 100 compounds were selected from the top scoring compounds that obeyed

Lipinski's rule of five for drug likeness and were from different structural clusters. Of the top 100 compounds, those that were commercially available were tested for their cytotoxic abilities in PC-3 and Du145 cells. Only six compounds, including LQZ-7, showed greater than 50% inhibition and were used in non-denaturing PAGE analysis for inhibition of survivin dimerization. LQZ-7 was able to dose dependently inhibit survivin dimerization and not interfere with another control homodimeric dimerization protein, 14-3-3σ. In a cell free translation experiment, LQZ-7 also inhibited the dimerization of nascent survivin protein. LQZ-7 is the primary hit utilized for the structure optimization experiments in the remaining sections.

4.2 Results

4.2.1 Generation of LQZ-7 Structural Analogues

The predicted binding modality of LQZ-7 using DOCK, involves the small molecule making critical hydrogen bonding interactions with the backbone of survivin. The first hydrogen bond is between the amine group positioned next to the methylated benzene ring and Glu^{94}. The second hydrogen bond is between the carboxylic acid group of LQZ-7 and Trp^{10} of survivin. The final significant interaction of the small molecule with survivin involves hydrophobic interactions between the furazanopyrazine ring and the key dimerization residues Leu^{98} and Phe^{101} (**Figure 6**) [61].

Our collaborator Dr. Mingji Dai at Purdue University graciously generated five structural analogues of LQZ-7 while trying to maintain or enhance the

interactions of the small molecules with the survivin backbone. Each analogue had the replacement of the oxadiazole moiety with a benzene ring that better fit in to the binding pocket in the survivin backbone. Each analogue also maintained the critical amine group and Glu^{94} hydrogen bonding interaction. The primary differences between the analogues and LQZ-7 was the replacement of the large bulky side chain and furazanopyrazine ring with moieties designed to better fit between the space between Leu^{98} and Phe^{101} with the idea in mind that the addition of atoms like fluorine may maintain key interactions and possibly provide a strong polar covalent bond between the small molecule and survivin (**Figure 16**).

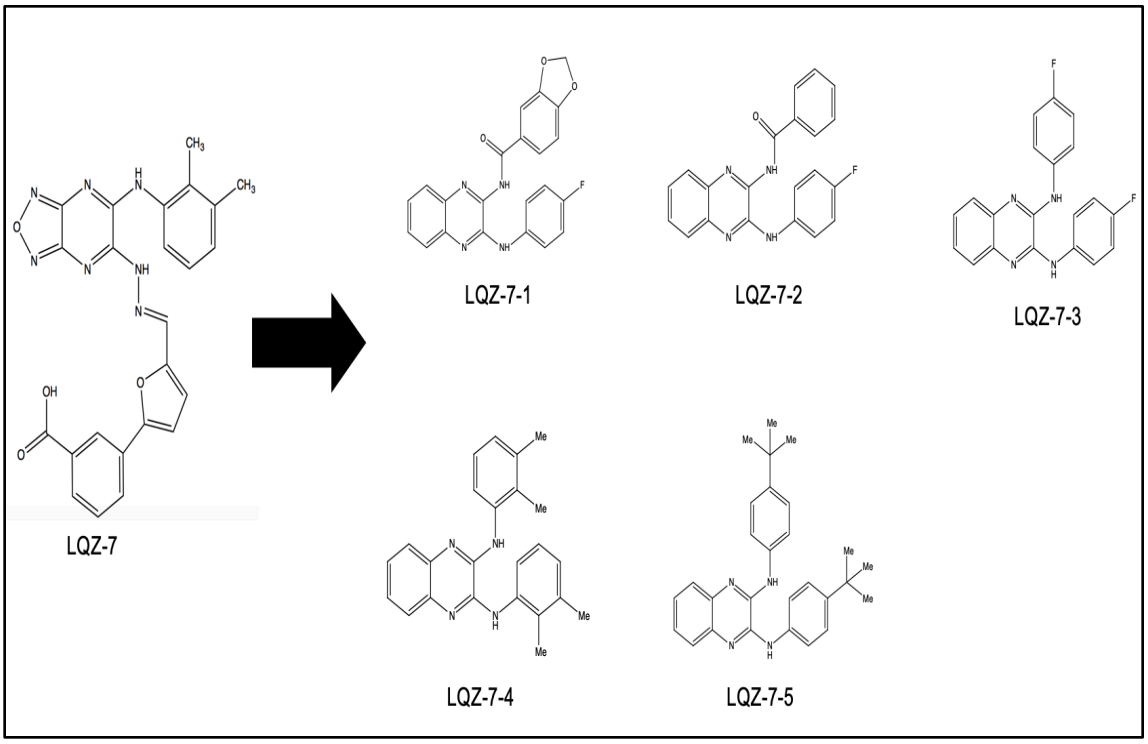


Figure 16. **Chemical structures of five analogues generated of LQZ-7.**

4.2.2 Analogues Cytotoxicity in Prostate Cancer Cells

After development and synthesis of the five LQZ-7 structural analogues, the first goal was to assess the cytotoxic nature of the analogues in relation to the parental LQZ-7 compound in prostate cancer cell lines, PC-3 and C4-2 by performing methylene blue experiments. Cells were tested over a concentration range of 0.1 μM to 40 μM. After seventy-two hour treatment, cells were fixed and percent cell viabilities were calculated. Interestingly in both PC-3 and C4-2 cells, only one structural analog, LQZ-7-3, showed enhanced potency as demonstrated by a lower IC_{50} value in the methylene blue assay (**Figure 17A and 17B**). LQZ-7-3 had an average IC_{50} value of 3-4.8 μM as compared 8-10 μM in the tested cell lines. LQZ-7-3 compound has the substitution of fluorinated benzene at the LQZ-7 amine position as well as the addition of a fluorine on the 1-benzene ring. The other four structural analogues had significantly higher IC_{50} values than that of the parental LQZ-7 compound. From this data LQZ-7-3 was selected as the new lead compound and was used in additional studies to characterize its mechanism of action and effect on a PC-3 xenograft tumor *in vivo*.

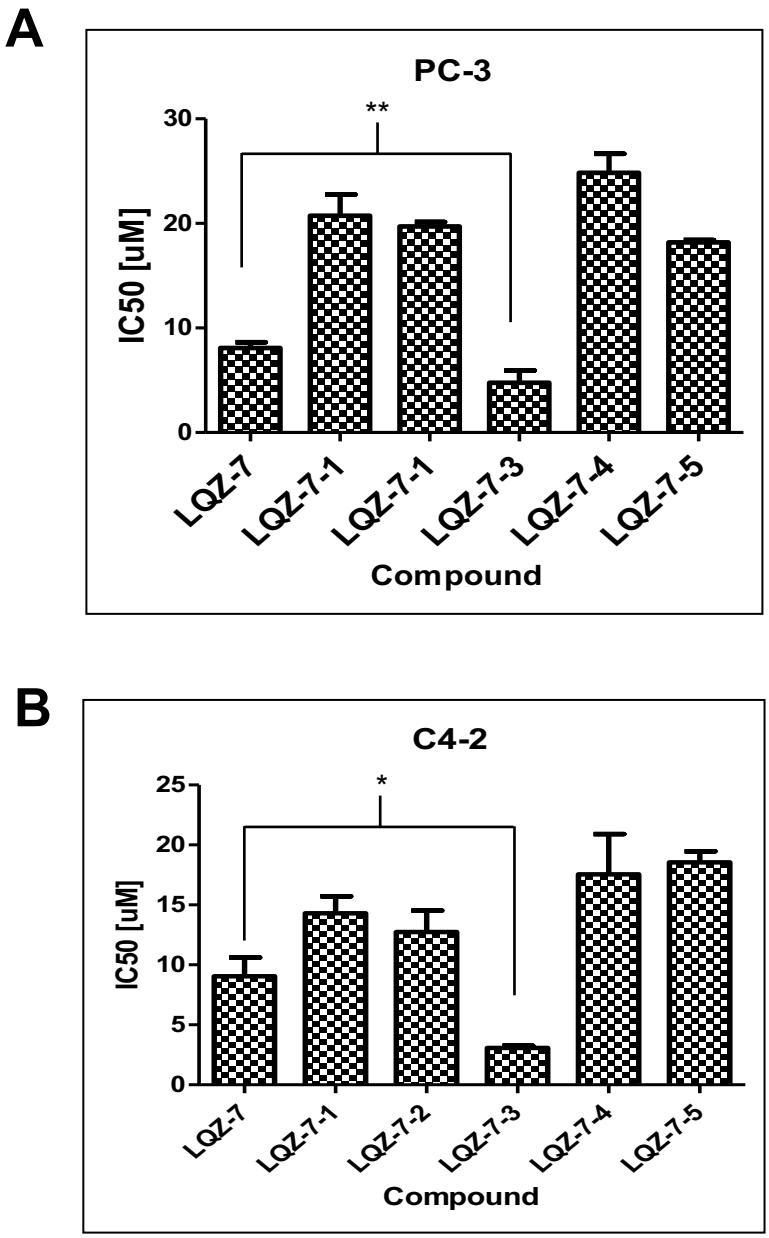
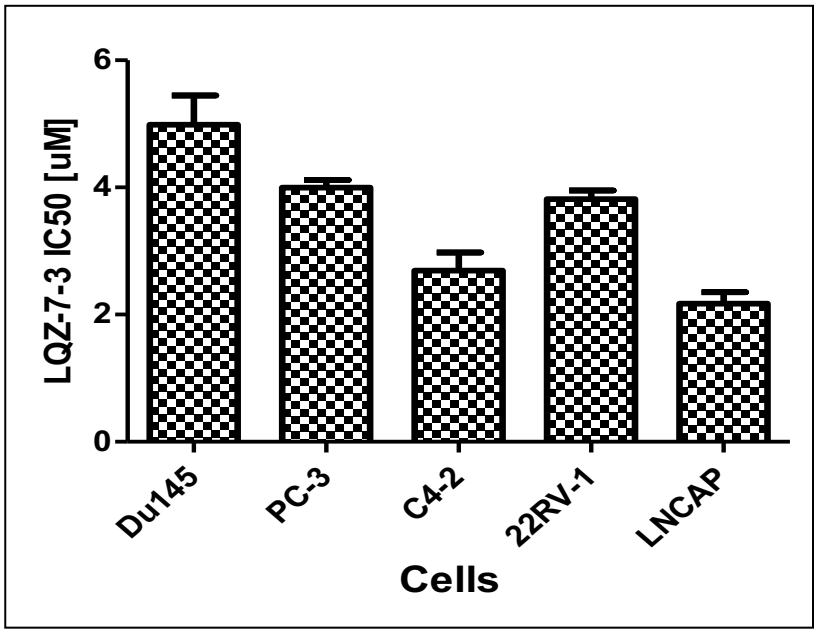


Figure 17. **Structural analogue LQZ-7-3 has enhanced cytotoxicity compared to LQZ-7.** One-Way ANOVA analysis of the average IC_{50} values of each compound tested in (A) PC-3 cells and (B) C4-2 cells. Each concentration was tested in triplicate. One Way ANOVA p-value = < 0.001. Post-Hoc Bonferroni Test ** = $p < 0.01$, * = $p < 0.05$. n = 3 independent experiments. Error bars equal standard deviation.

4.2.3 Correlation Analysis of Survivin Expression and LQZ-7-3 Cytotoxicity

To assess the activity and selectivity towards survivin, I next performed a correlation analysis of survivin expression in five prostate cancer cell lines and the average LQZ-7-3 IC_{50} in each cell line. As previously shown in **Figure 8**, the four castration resistant cell lines had a higher survivin level than that of the androgen dependent LNCAP cell line. Similar results were seen when the IC_{50} of LQZ-7-3 was determined by methylene blue in each of the lines (**Figure 18A**). As shown in the correlation analysis (**Figure 18B**), the IC_{50} values strongly positively associate with the survivin protein level in these cell lines with a coefficient of determination of 0.79, indicating that LQZ-7-3 likely also suppresses the survival of these cancer cells through its action on survivin.

A



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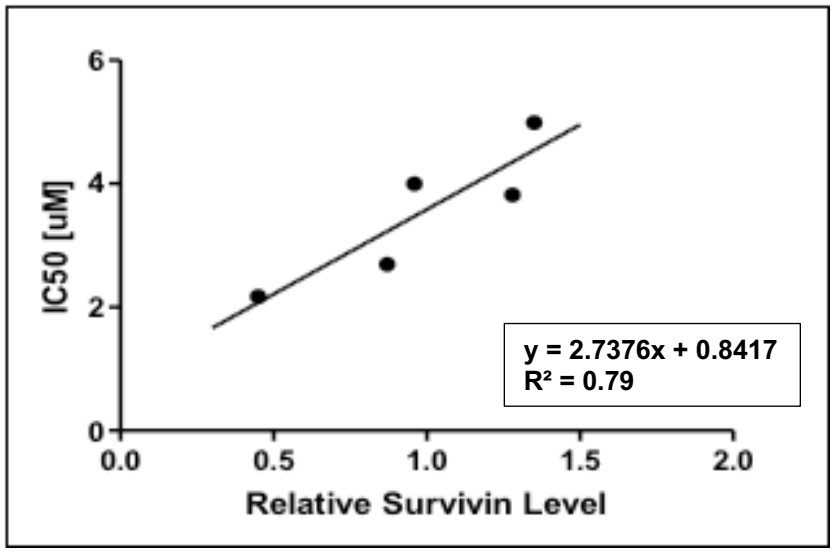


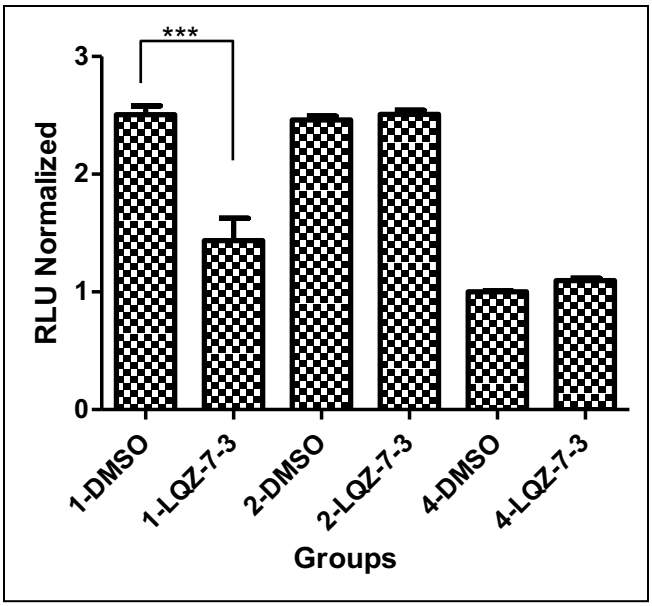
Figure 18. **Survivin protein level correlates with LQZ-7-3 IC_{50}.** (A) Average IC_{50} of methylene blue analysis of five prostate cancer cell lines using analogue LQZ-7-3. Each concentration was tested in triplicate (B) Correlation analysis of relative survivin level vs docetaxel IC_{50}. Strong positive $R^2 = 0.79$. n = 3 independent experiments.

4.2.4 Mammalian Two Hybrid Assay with Analogues

As this class of survivin inhibitors are designed to be exerting their action through interaction and disruption of survivin dimerization, I assessed LQZ-7-3 ability to diminish survivin dimerization in an *in vitro* mammalian two hybrid assay. This assay assesses the ability of two proteins to interact and bring together the GAL4-DNA Binding Domain of one plasmid and the activation domain of another plasmid to turn on the transcription of Secreted Embryonic Alkaline Phosphatase (SEAP) reporter gene. SEAP catalyzes the hydrolysis of p-Nitrophenyl phosphate to produce a yellow end product that can be measured in the collected culture media and read spectrophotometrically at 405 nm.

As shown in **Figure 19A**, LQZ-7-3 significantly decreased the SEAP gene production in the group 1 set of plasmids containing each plasmid with the survivin coding region insert as compared to the basal level of SEAP in cells shown by group 4. LQZ-7-3 also had no effect on the pM-53/pVP16-T group 2 positive control. To determine if LQZ-7-3 decreased survivin dimerization greater than parental compound LQZ-7, I performed a similar mammalian two-hybrid assay. Whereas, both LQZ-7 and LQZ-7-3 decreased group 1 survivin containing plasmids interaction, LQZ-7-3 decreased survivin dimerization significantly greater than LQZ-7 (**Figure 19B**). LQZ-7-3 also decreased group 1 survivin plasmid dimerization greater than the other LQZ-7 analogues (**Figure 19C**). These findings provide additional evidence that LQZ-7-3 is working through disruption of survivin dimerization.

A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RLU Normalized
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4-DMSO
4-LQZ-7-3
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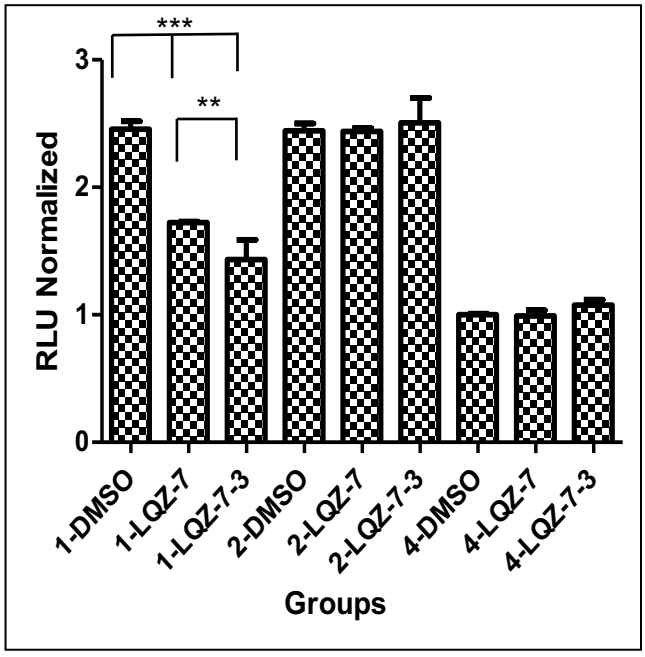
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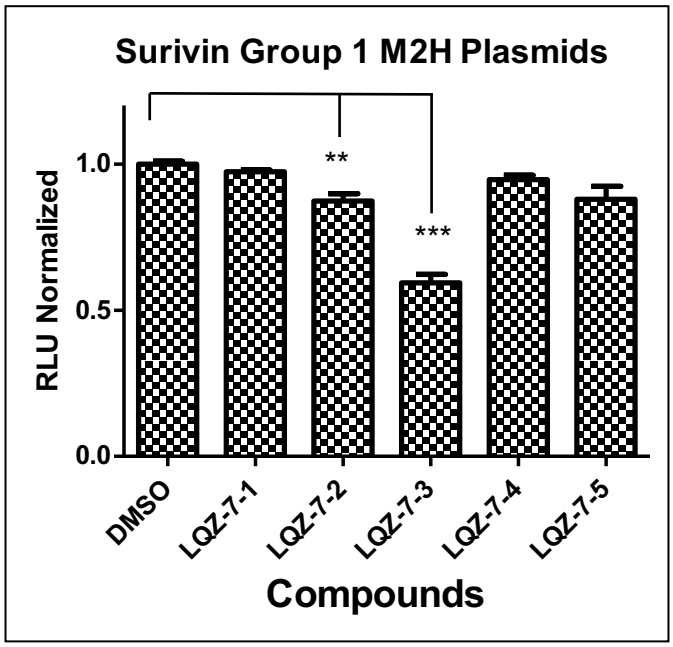


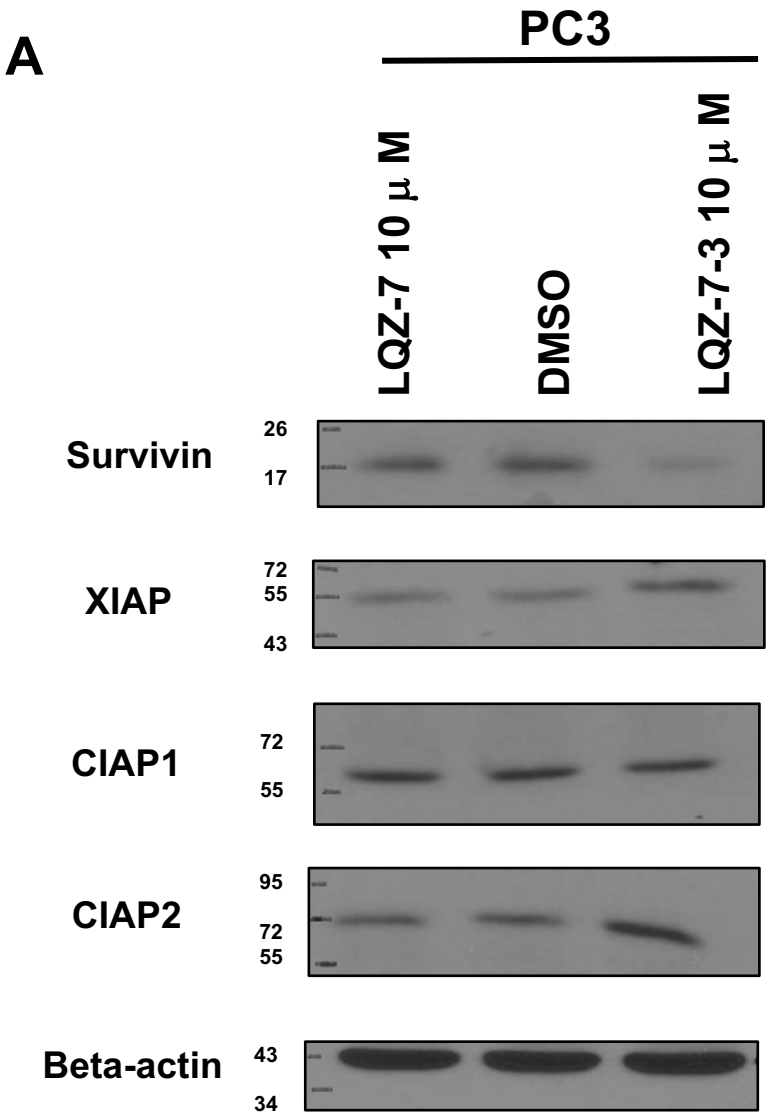
Figure 19. **LQZ-7-3 disrupts survivin dimerization to a greater extent than LQZ-7.** (A) Mammalian two hybrid survivin dimerization assay showing decreased RLU value in the cells transfected with the survivin plasmids (Group 1) and treated with LQZ-7-3. Group 2 is a positive control for SEAP that involves interaction of pM-53 and pVP16-T plasmids. Group 4 are empty vector transfections to determine the basal level of SEAP in the cell media. *** = P-value < 0.001. n = 3 independent experiments. (B) Mammalian two hybrid survivin dimerization assay showing decreased RLU value in the cells transfected with the survivin plasmids (group 1) and treated with LQZ-7-3 to a greater extent than those treated with LQZ-7. *** = P-value < 0.001. ** = p-value < 0.01. n = 3 independent experiments. (C) Effect of analogues 1-5 on Group 1 Survivin plasmids in M2H assay only. *** = P-value < 0.001. ** = p-value < 0.01. n = 3 independent experiments. Error bars equal standard deviation. Each transfection was performed in triplicate.

4.2.5 Survivin and IAP Family Members Degradation Assay

To further establish LQZ-7-3 selectivity towards survivin, the effect of LQZ-7-3 treatment on survivin and other IAP Family member proteins level in prostate cancer cells was assessed by western blot analysis. As shown in **Figure 20A and 20B**, 48 hour treatment with LQZ-7-3 decreased survivin protein level as compared to DMSO control in both PC-3 and C4-2 cells. LQZ-7-3 also decreased survivin protein level more than cells treated with LQZ-7 compound. Interestingly, despite being in the same protein family and sharing similar domain features, LQZ-7-3 treatment did not change the expression level of other Inhibitors of Apoptosis: XIAP, CIAP1, or CIAP2. These results give additional evidence that the small molecule inhibitors targeting survivin have specificity for the intended target protein versus other BIR domain containing proteins.

4.2.6 Survivin Stable Overexpression Effect on LQZ-7-3 Cytotoxicity

To determine if overexpression of survivin would alter the sensitivity of cancer cells to LQZ-7-3, the stable transfected an HA-Tagged Survivin C4-2 cells from previous studies were utilized in this experiment. To evaluate the effect of this overexpression on the sensitivity of these cells to LQZ-7-3, once again a methylene blue assay was performed. As shown in **Figure 21,** C4-2 Survivin cells had a significantly higher IC_{50} at roughly 7 μM as compared to C4-2 pcDNA3.1 vector cells that had an IC_{50} of 2 μM. The data indicate that overexpression of survivin decreases sensitivity to LQZ-7-3 as expected.


A
PC3
LQZ-7 10 μ M
DMSO
LQZ-7-3 10 μ M
Survivin
26
17
XIAP
72
55
43
CIAP1
72
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CIAP2
95
72
55
Beta-actin
43
34

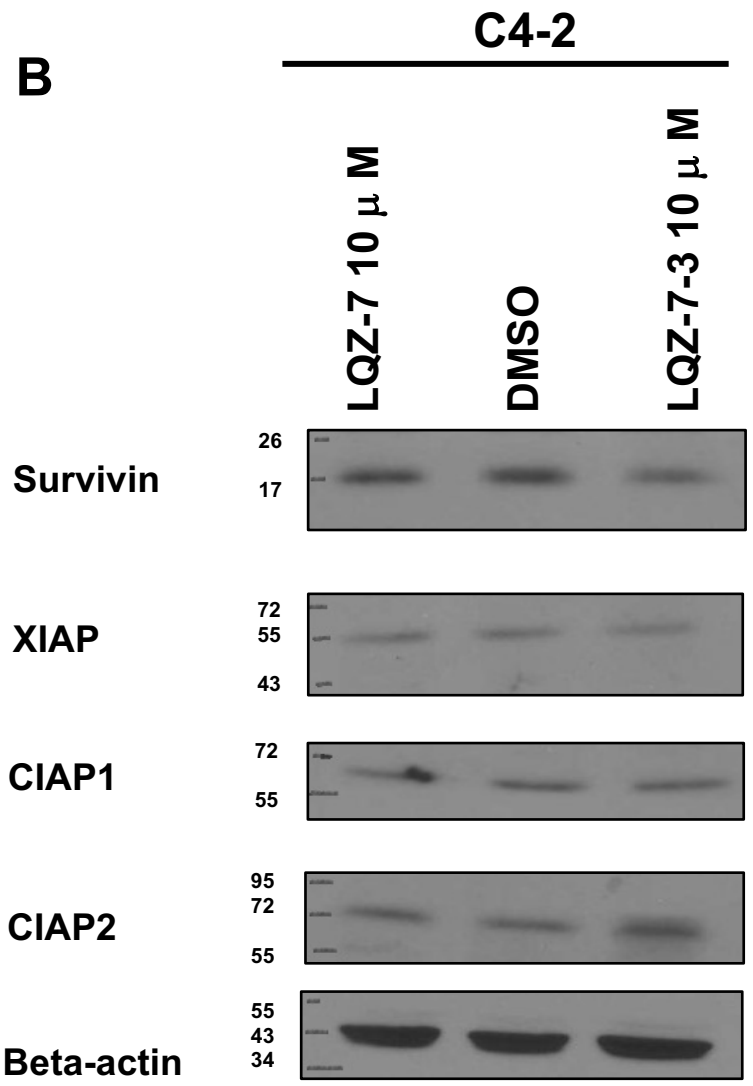


Figure 20. **LQZ-7-3 decreases survivin level without altering other IAP family members levels.** Western blot analysis for survivin protein level and other IAP protein level after treatment with LQZ-7 and LQZ-7-3 in (A) PC-3 cells and (B) C4-2 cells. Survivin is decreased at 48 hours with treatment with both survivin inhibitors but levels of other IAP family members do not decrease. LQZ-7-3 decreases survivin level greater than LQZ-7 treatment. n = 3 independent experiments.

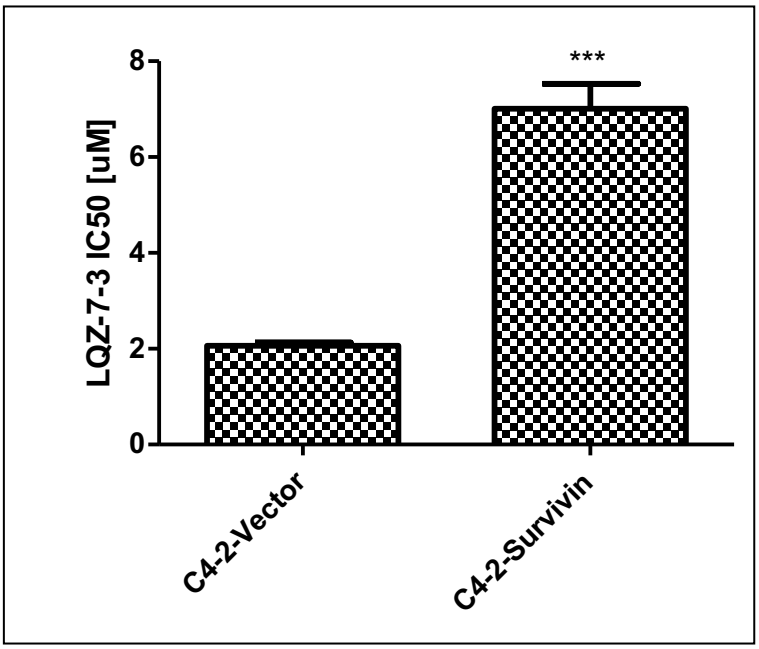


Figure 21. **Survivin stable overexpression decreases cancer cells sensitivity to LQZ-7-3.** Overexpression of survivin results in a higher IC_{50} value for the LQZ-7-3 compound in a C4-2-survivin stable overexpression cells compared to vector transfected cells in a methylene blue assay. Each concentration was tested in triplicate. *** = p-value < 0.001. n = 3 independent experiments.

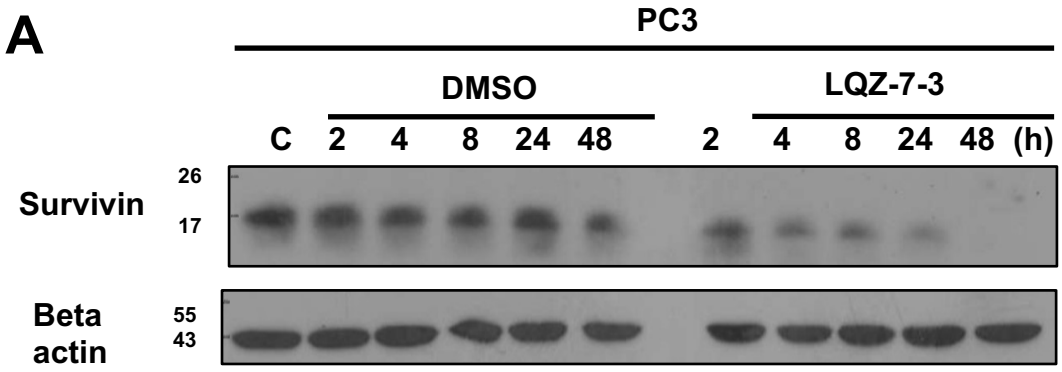
4.2.7 LQZ-7-3 Survivin Degradation Time Course

In the previous degradation assay, LQZ-7-3 treatment led to survivin loss at 48 hours compared to DMSO treated cells. In order to determine the time at which there is a clear difference in survivin protein level after LQZ-7-3 treatment compared to DMSO control treated cells a degradation timepoint assay was performed. Both PC-3 and C4-2 cells were treated for 0, 2, 4, 8, 24, 48 hours with DMSO control or 10 μM LQZ-7-3 followed by western blotting analysis for survivin. It is important to note that survivin protein level can be heavily influenced by cell-cell contact, thus careful consideration was given to the cell density plated to ensure that no timepoint (particularly 48 hrs.) used >85% confluent cells. In PC-3 cells, survivin protein level had decreased as early as 2 hours whereas the decrease in survivin protein level was not seen until 8 hours in C4-2 cells (**Figure 22A and 22B**). The most significant differences in the time course came at 24 hours and 48 hours for both cell lines. In total, LQZ-7-3 results in rapid loss of survivin protein in prostate cancer cells.

4.2.8 LQZ-7-3 Effect on Survivin Half-Life

Survivin is a dynamic protein with a short half-life in the cell. To assess if LQZ-7-3 treatment could result in a change in the half-life of survivin compared to DMSO treated cells, a cycloheximide assay was utilized. To this end, PC-3 and C4-2 cells were pretreated with cycloheximide for one hour to inhibit synthesis of new proteins followed by chasing with LQZ-7-3 or DMSO for different times. Cycloheximide remained in the culture the duration of the experiment. Similarly to

the time course cells were used at roughly 65-75% confluency to avoid dramatic increases in survivin expression associated with cell-cell contact. **Figures 23A and 23B** show that the half-life of DMSO control treated PC-3 cells is 2.27 hours and 2.17 hours in C4-2, which is consistent with previously reported half-life of survivin [209]. However, after treatment with LQZ-7-3 the half-life of survivin was 22 minutes in PC-3 cells and 50 minutes in C4-2 cells. The data in this section illustrates LQZ-7-3 treatment results in a significant decrease in the half-life of survivin in prostate cancer cells.


A
PC3
DMSO
LQZ-7-3
C 2 4 8 24 48 2 4 8 24 48 (h)
Survivin
26
17
Beta actin
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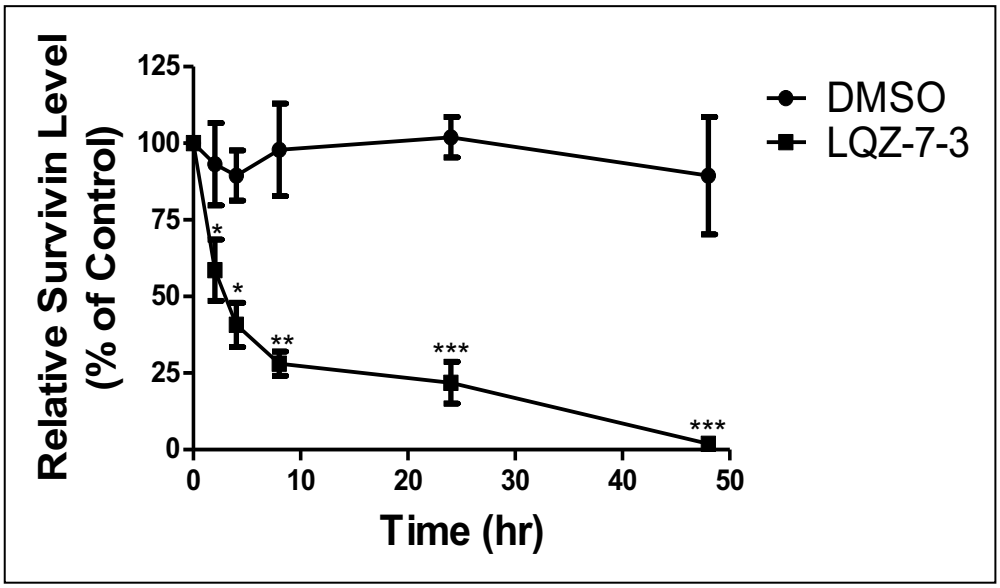
43


Relative Survivin Level (% of Control)
125
100
75
50
25
0
0 10 20 30 40 50
Time (hr)
DMSO
LQZ-7-3
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B

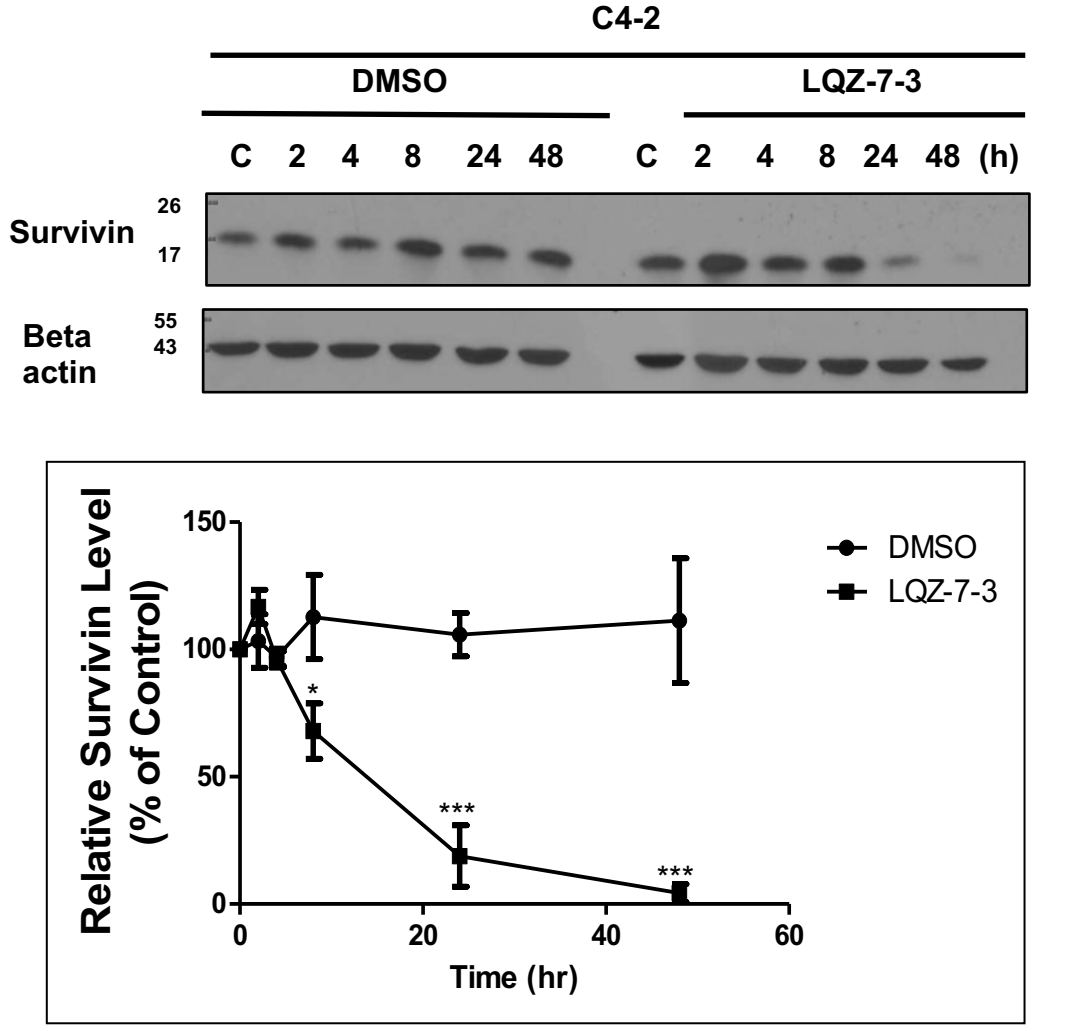
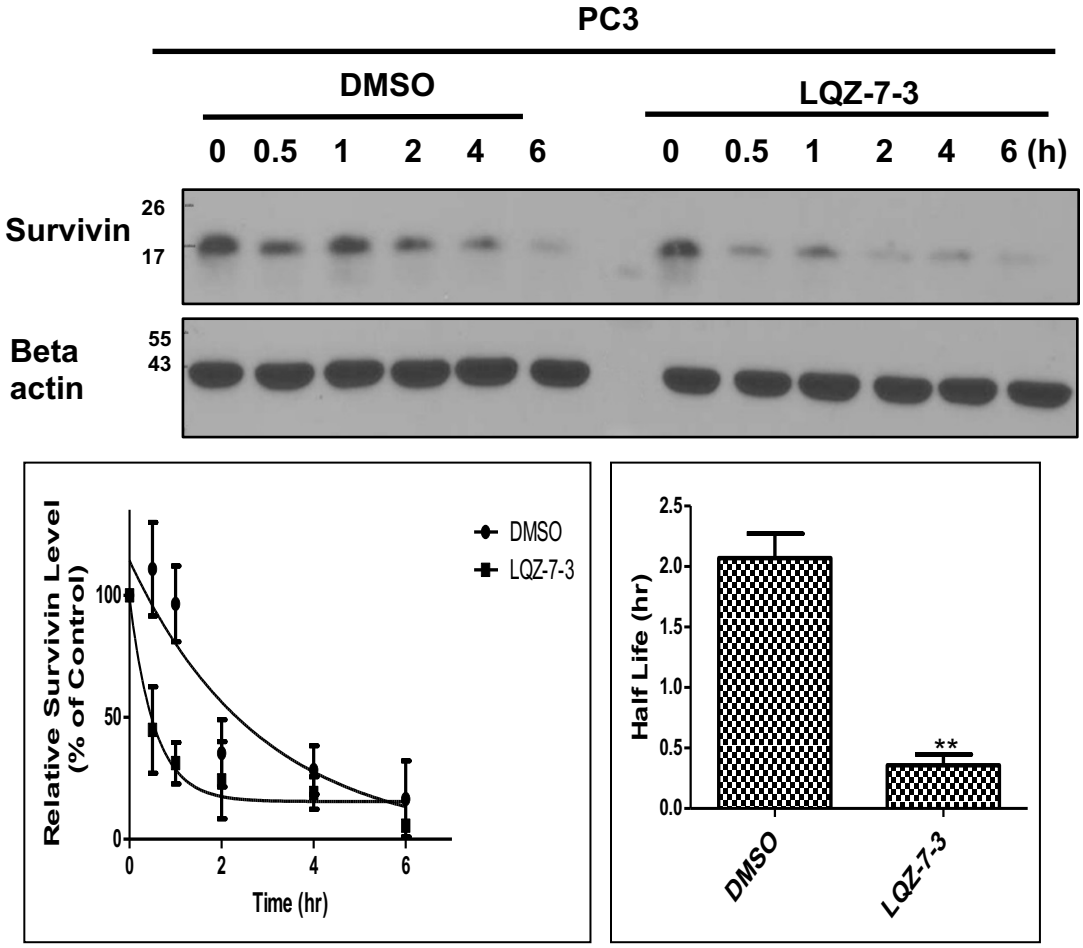


Figure 22. **LQZ-7-3 treatment decreases survivin protein level rapidly in prostate cancer cells.** Time course of LQZ-7-3 treatment effect on survivin protein level at 0, 2, 4, 8, 24, 48 hours in (A) PC-3 cells and (B) C4-2 cells. * = p-value < 0.05. *** = p-value < 0.001. n = 3 independent experiments.


A
PC3
DMSO
LQZ-7-3
0 0.5 1 2 4 6
0 0.5 1 2 4 6 (h)
Survivin
26
17
Beta actin
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43
Relative Survivin Level (% of Control)
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Time (hr)
DMSO
LQZ-7-3
Half Life (hr)
2.5
2.0
1.5
1.0
0.5
0.0
**
DMSO
LQZ-7-3

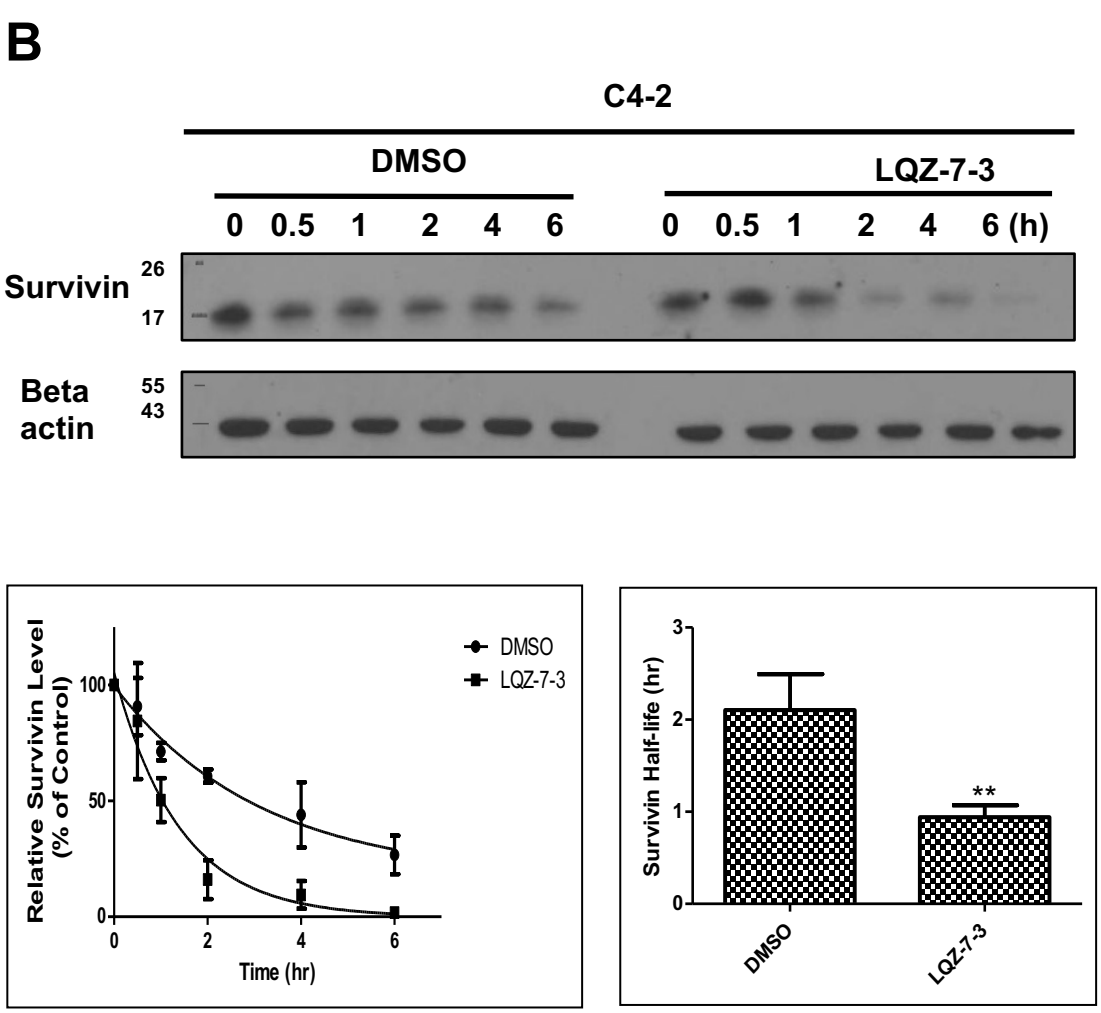


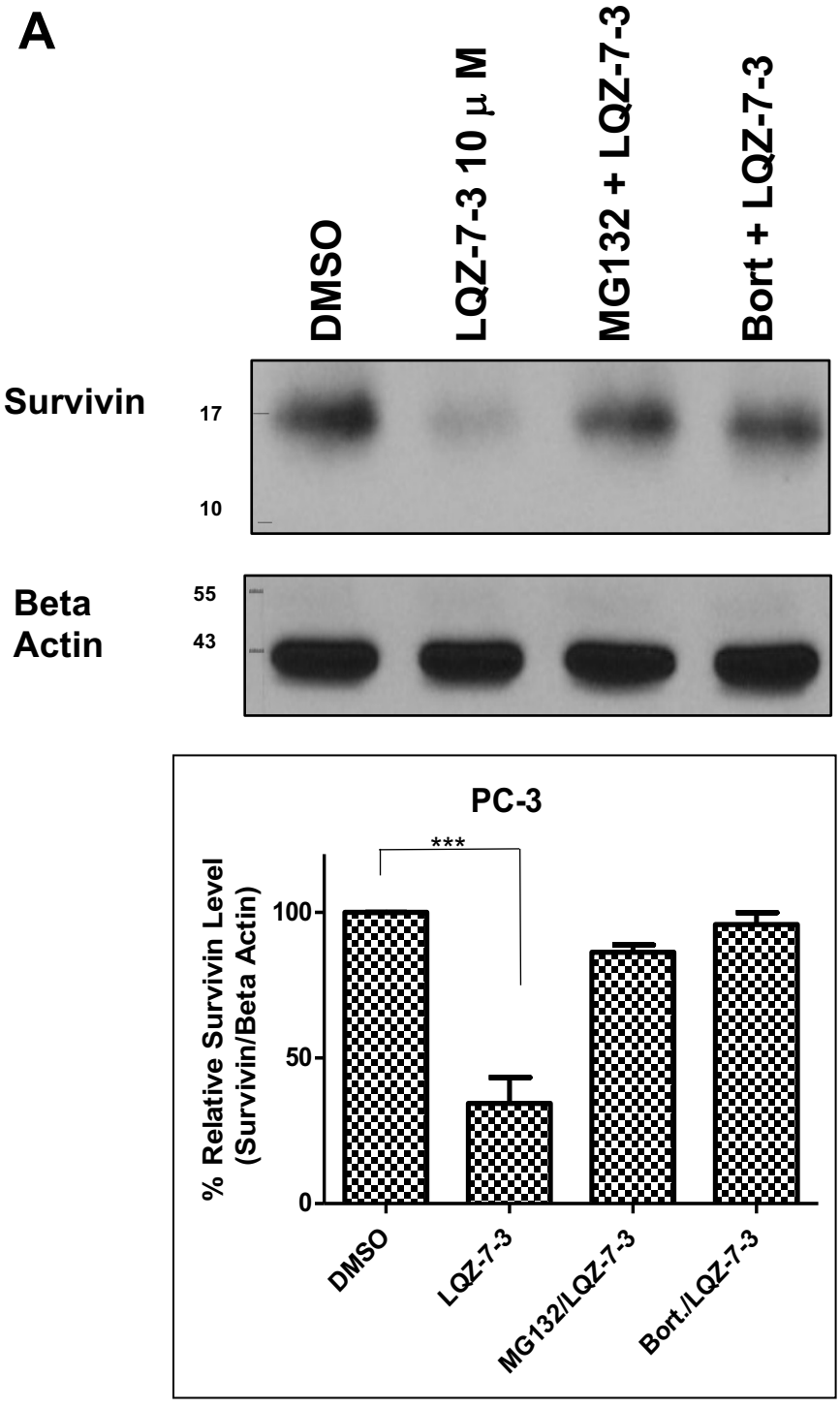
Figure 23. **LQZ-7-3 treatment decreases survivin protein half-life in prostate cancer cells.** Survivin half-life determined by cycloheximide assay in DMSO control and LQZ-7-3 treated (A) PC-3 and (B) C4-2 cells. Cells were pretreated with cycloheximide for one hour then chased with DMSO or LQZ-7-3 for different times. The curved graph represents the average exponential decay curve of three independent experiments. ** = p-value < 0.01. n = 3 independent experiments.

4.2.9 LQZ-7-3 and Proteasome Inhibitor Rescue Experiment

To determine if LQZ-7-3 promotes survivin degradation via proteasome, PC-3 and C4-2 cells were pretreated with two different proteasome inhibitors MG132 and bortezomib for two hours before treatment with the compound. As expected LQZ-7-3 reduced survivin level significantly as compared to DMSO control (**Figure 24A and 24B**). Interestingly, pretreatment with the proteasome inhibitors rescued the survivin to a similar level as seen in the DMSO treatment group. Thus, LQZ-7-3 likely promotes survivin degradation via the proteasome.

4.2.10 Effect of LQZ-7-3 Treatment on PC-3 Cell Cycle

To asses if LQZ-7-3 treatment has an effect on the cell cycle of prostate cancer cells, a propidium iodide flow cytometry study was performed. Specifically, PC-3 cells were treated with the roughly the IC_{50} of LQZ-7-3 (4μM) for 48 hours and subsequently stained with propidium iodide and analyzed by flow cytometry. It is important to note this experiment was only performed a single time, but treatment with LQZ-7-3 in the PC-3 cell line did not show a discernible effect on the cell cycle compared to DMSO treated cells (**Figure 25**). Despite the limited scale and scope of this experiment, it did guide us to move on from cell cycle changes and to other potential mechanism for LQZ-7-3 activity, such as apoptosis.


A
DMSO
LQZ-7-3 10 μ M
MG132 + LQZ-7-3
Bort + LQZ-7-3
Survivin
17
10
Beta Actin
55
43
PC-3
***
% Relative Survivin Level (Survivin/Beta Actin)
100
50
0
DMSO
LQZ-7-3
MG132/LQZ-7-3
Bort./LQZ-7-3

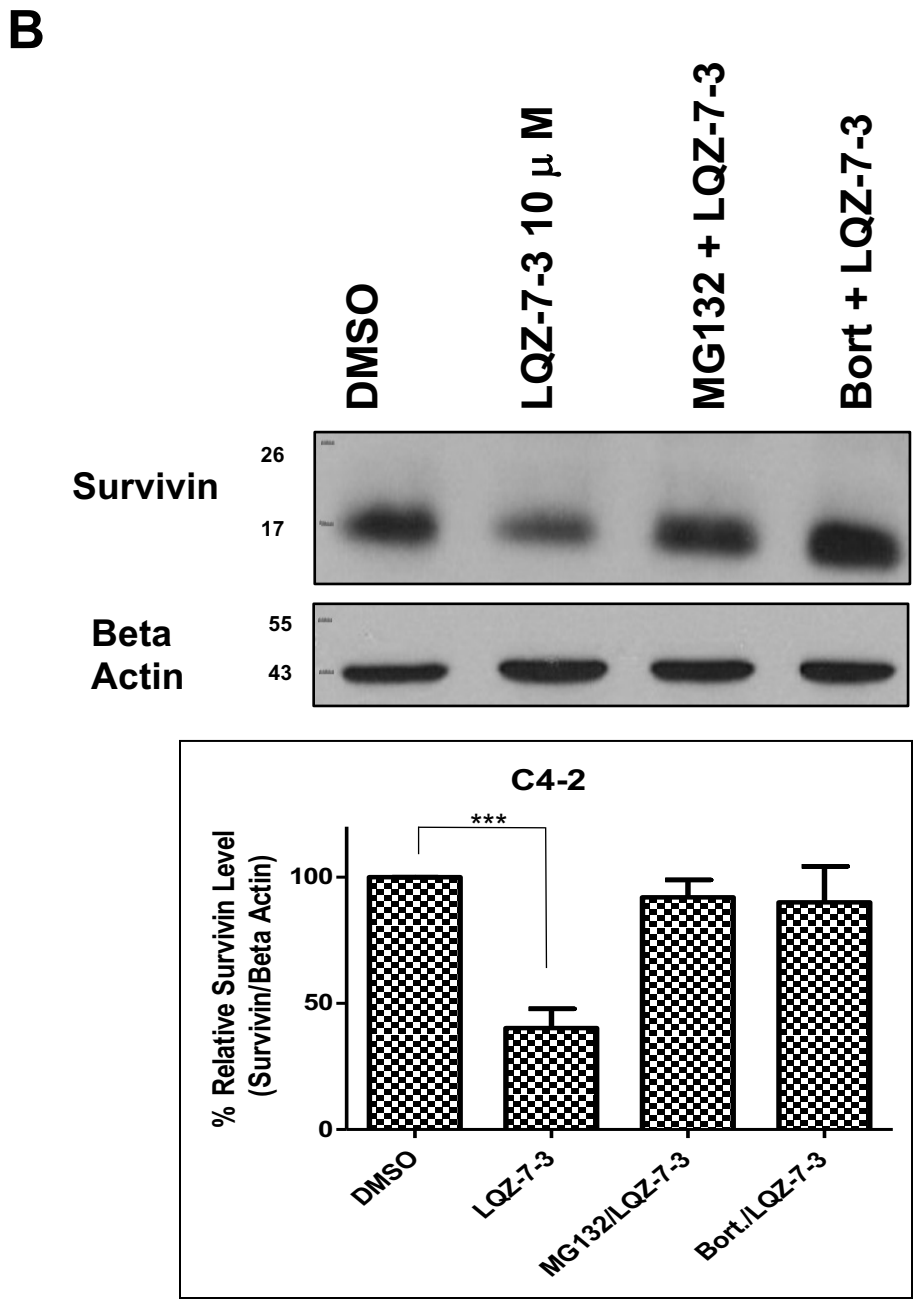


Figure 24. **Pretreatment with proteasome inhibitors rescues LQZ-7-3 induced survivin degradation.** Survivin loss caused by 24 hour treatment with LQZ-7-3 was able to be restored by blocking proteasome activity via pretreatment with two different inhibitors 7 μmol/L MG132, or 70 nmol/L bortezomib and incubation for 2 hours in (A) PC-3 and (B) C4-2 cells. Survivin level was nearly completely rescued to DMSO control level. *** = p-value < 0.001. n = 3 independent experiments. Error bars equal standard deviation.

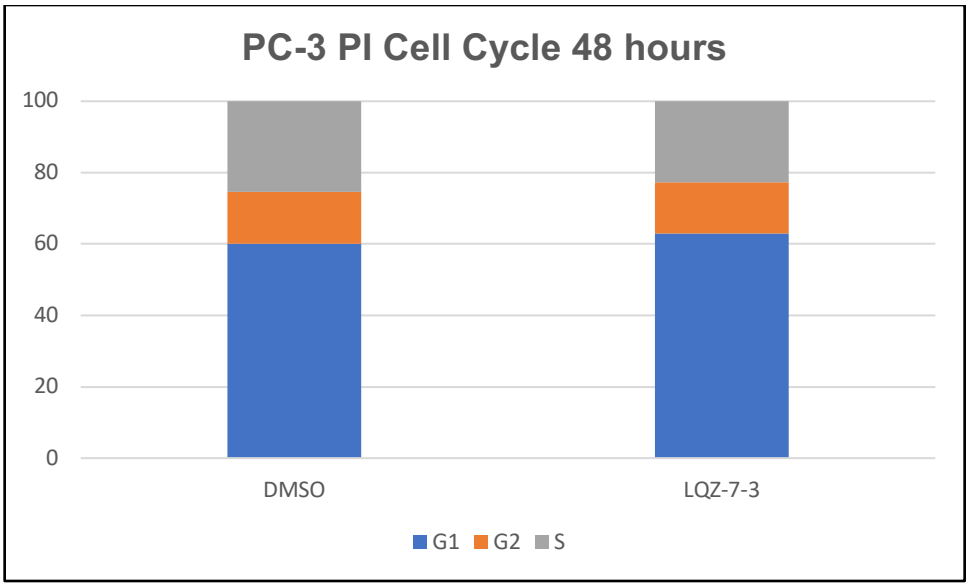
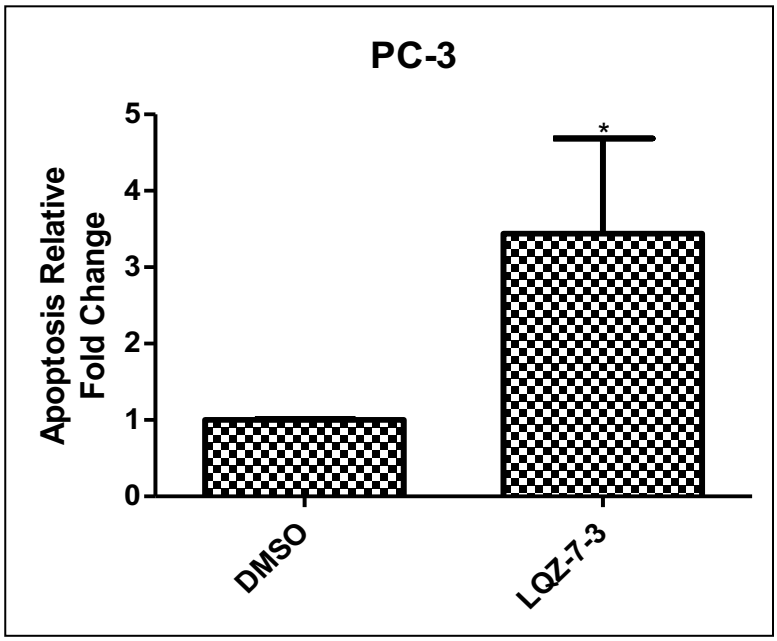


Figure 25. **LQZ-7-3 treatment does not change PC-3 cell cycle status.** Cell cycle differences between DMSO and LQZ-7-3 4 μM treated PC-3 cells was assessed at 48 hours via propidium iodide staining and flow cytometry. Despite being a single experiment no cell cycle differences were evident between the two treatment conditions. n = 1 experiments.

4.2.11 LQZ-7-3 induces spontaneous apoptosis

Since it has been previously demonstrated that dominant negative forms of survivin may cause spontaneous apoptosis of cancer cells [126] I next tested if LQZ-7-3 also induces apoptosis through its inhibition of survivin. To determine if LQZ-7-3 induces apoptosis of cancer cells Flow Cytometry with Annexin V staining was performed. Treatment with 3 μM LQZ-7-3 generated 3.5 and 19.03 relative fold increase in apoptosis in PC-3 and C4-2 cells respectively (**Figure 26A and 26B)**. In order to validate the apoptosis data from the annexin v staining, western blotting analysis was performed utilizing an apoptosis marker, cleaved caspase 3, which is activated and protein levels increase during the apoptosis cascade. As shown in **Figures 27A and 27B**, treatment with LQZ-7-3 caused a dose dependent increase in cleaved caspase 3 levels in both PC-3 and C4-2 cells. Overall this section provides evidence that LQZ-7-3 promotes cancer cell death via apoptosis.

A



B

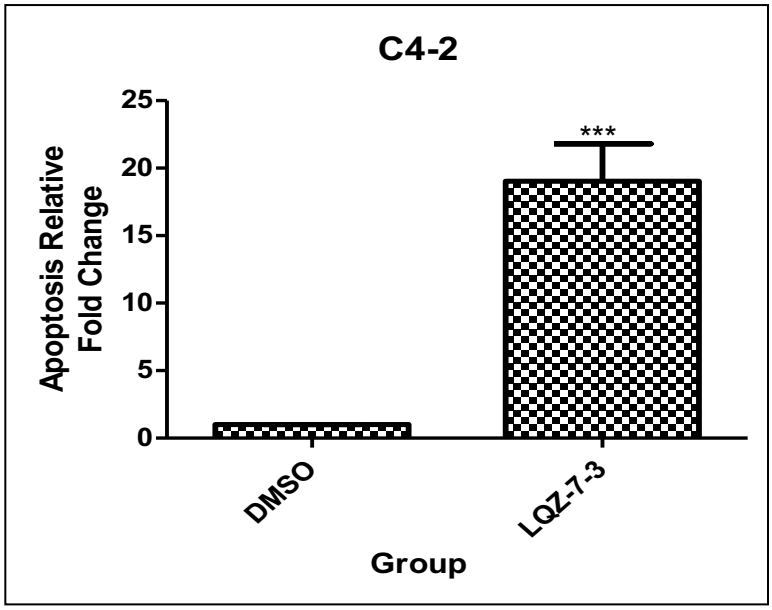
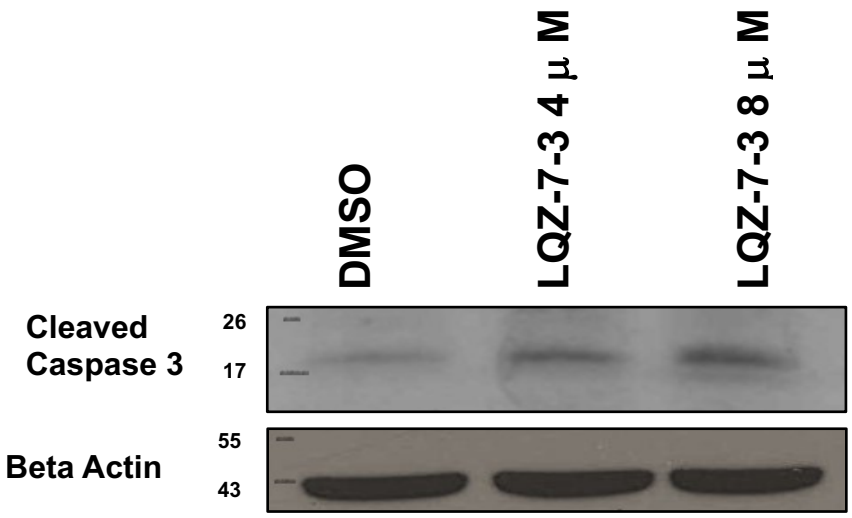
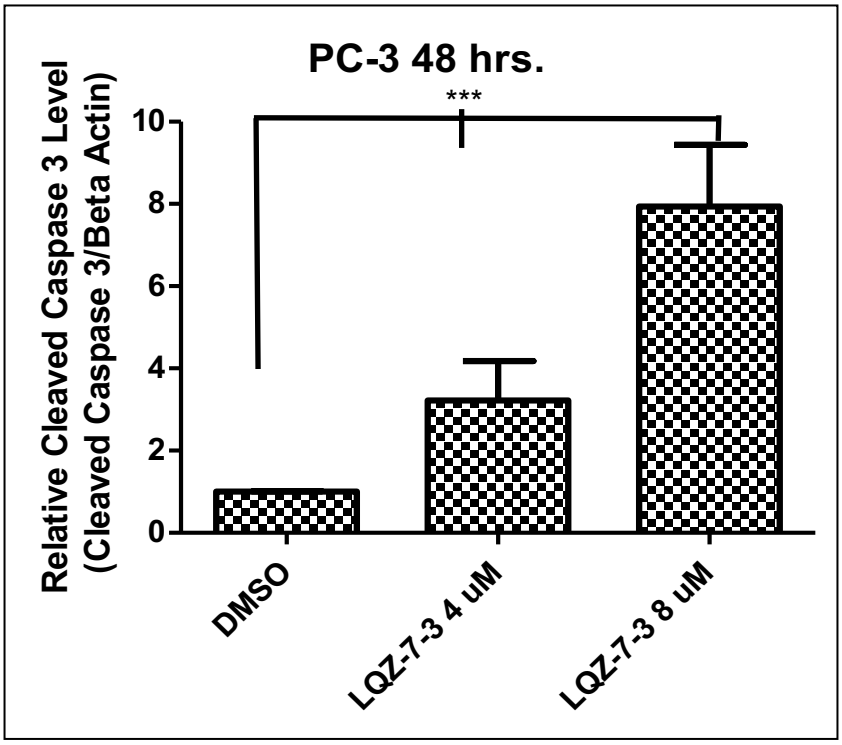


Figure 26. **LQZ-7-3 treatment causes increased apoptosis in prostate cancer cells.** Survivin loss caused by treatment with LQZ-7-3 causes increased apoptosis in (A) PC-3 and (B) C4-2 cells as evidenced by increased Annexin V staining Flow Cytometry. *** = p-value < 0.001. n = 3 independent experiments. Error bars equal standard deviation.

A


DMSO
LQZ-7-3 4 μ M
LQZ-7-3 8 μ M
Cleaved Caspase 3
26
17
Beta Actin
55
43


PC-3 48 hrs.
***
Relative Cleaved Caspase 3 Level (Cleaved Caspase 3/Beta Actin)
10
8
6
4
2
0
DMSO
LQZ-7-3 4 uM
LQZ-7-3 8 uM

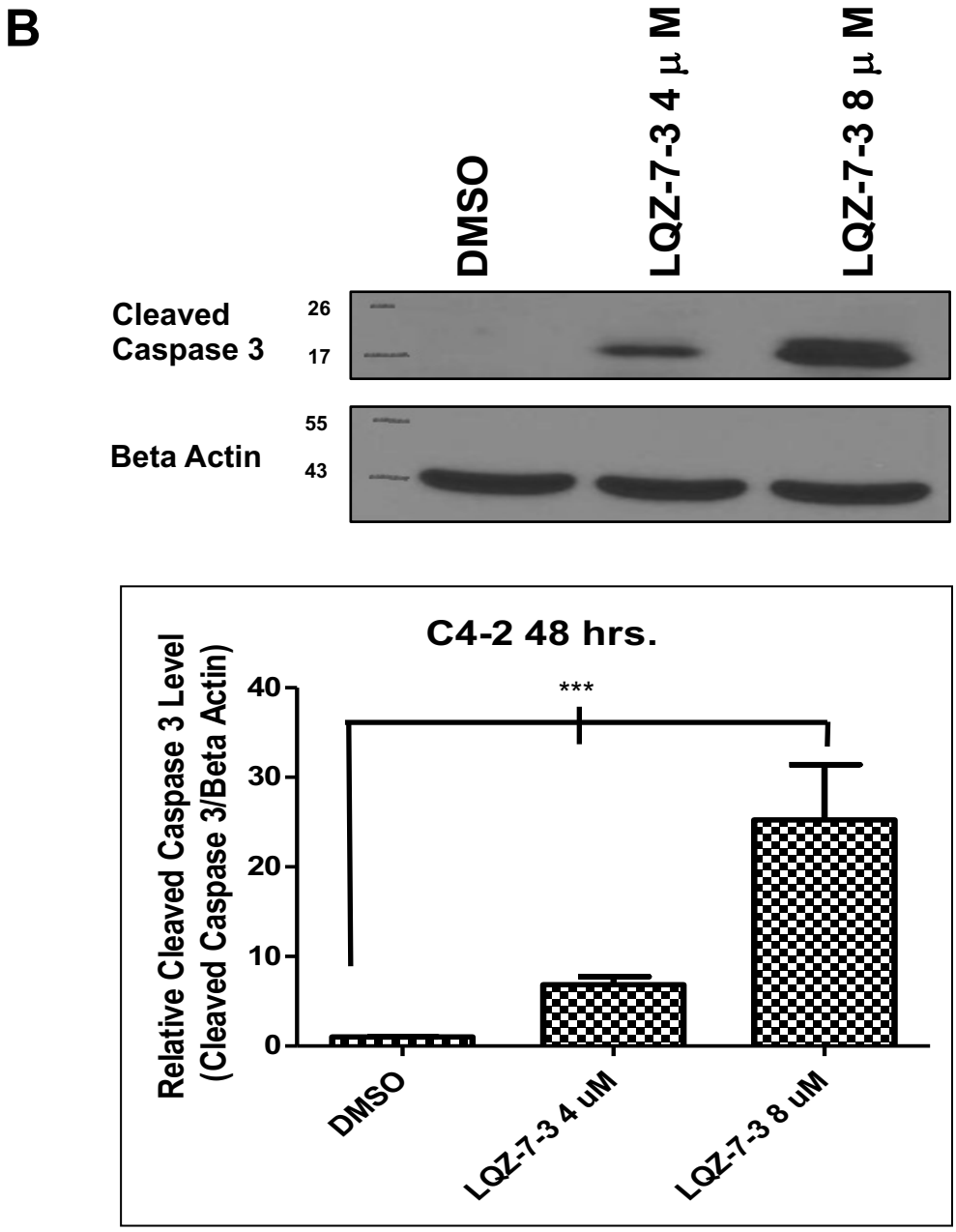


Figure 27. **LQZ-7-3 treatment causes increased cleaved caspase 3 in cancer cells.** Treatment with LQZ-7-3 leads to a dose dependent increase in cleaved caspase 3 levels as seen by western blot analysis in (A) PC-3 and (B) C4-2 cells. The increase in cleaved caspase 3 is confirmation of the flow cytometry data indicating increased apoptosis after LQZ-7-3 treatment. *** = p-value < 0.001. n = 3 independent experiments. Error bars equal standard deviation.

4.2.12 Solubility, *In Vivo* Toxicity, and Efficacy of LQZ-7-3

In order to determine if LQZ-7-3 is orally available and efficacious as an anti-cancer agent, a PC-3 xenograft mouse model study was performed. However, before this work could proceed I sought to identify a suitable formulation for delivering LQZ-7-3 orally *in vivo*.

4.2.12.1 Solubility

To determine the most suitable formulation for LQZ-7-3 to be given via oral gavage, multiple different solvents and combinations were tested. The different solvents tested to solubilize LQZ-7-3 included water, PBS, and multiple different oils. Eventually it was determined that a 90% corn oil and 10% DMSO best solubilized LQZ-7-3 up to 20 mg/mL without particulate formation. To make the stock solution for LQZ-7-3, the compound is first solubilized with 10% DMSO followed by the addition of the 90% corn oil.

4.2.12.2 Acute Toxicity Study

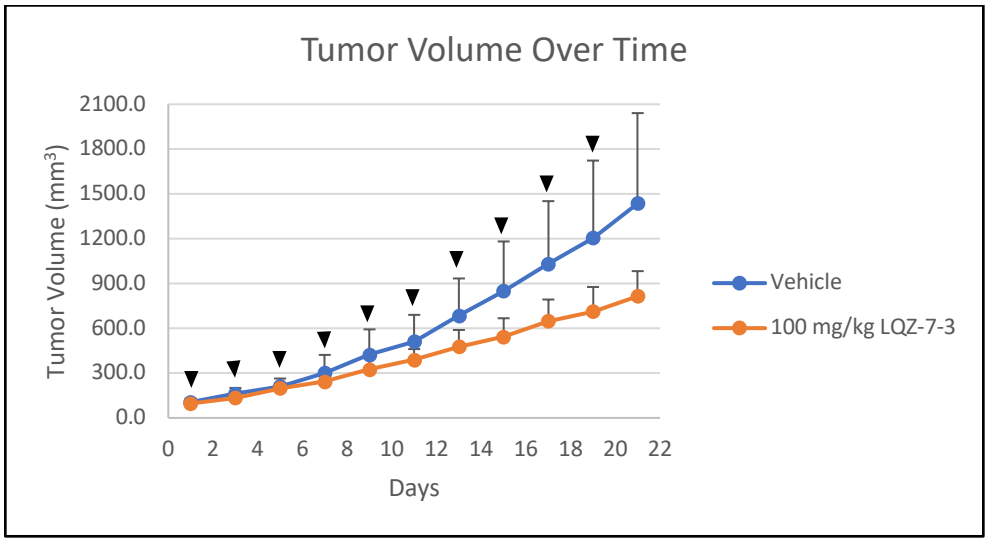
Before beginning an *in vivo* efficacy study, safety profile of our LQZ-7-3 corn oil/DMSO oral formulation was assessed by performing an acute toxicity study. The highest dose a mouse received was 200mg/kg for a total of four treatments every other day. This mouse showed no adverse effects or loss in body weight during the time of the study. This study was performed in such a manner as to simulate the dosing interval that would be used in a future efficacy study. The toxicity experiment was also meant to elucidate any dangers involved in this dosing strategy and the timeframe was designed to represent roughly the

half-way mark of a proposed efficacy study where decisions could be made to progress or end the study.

4.2.12.3 LQZ-7-3 Efficacy Study

To determine if LQZ-7-3 is orally active in suppressing tumor growth, an *in vivo* PC-3 xenograft model was used in an efficacy study. For the efficacy study, 3 x 10^6 PC-3 cells were first implanted subcutaneously in the hind flanks of six-week old male NSG mice to establish xenograft tumors. When the tumor volume reached approximately ~100mm^3 the mice were randomized, with five mice per group and treated with vehicle control (90% corn oil/10% DMSO) or 100 mg/kg LQZ-7-3 via oral gavage every other day for a total of ten treatments. As shown in **Figure 28**, at the end of treatment LQZ-7-3 treatment group had a significant decrease in tumor volume. Although the tumor dry weight was not significant, LQZ-7-3 treatment group trended towards a decrease in tumor dry weight at the end of the study compared to vehicle group (**Figure 29**). Tumors in the LQZ-7-3 treatment group tended to be smaller and less red in color as compared to those in the vehicle group (**Figure 30**). Importantly, throughout the study I did not see any adverse effects with mice remaining active, no visible signs of wasting, or changes in organ weight amongst the two groups. The bodyweight of mice remained constant with vehicle group after multiple rounds of dosing of LQZ-7-3 suggesting that LQZ-7-3 doesn't cause major toxicities (**Figure 31A and 31B**). These observations were similar to those seen in the short acute toxicity study that was performed before the efficacy study where I were able to perform multiple dosing up to 200mg/kg without decreases in body weight or adverse

effects. Western blot analysis of dissected xenograft tumors showed that the average survivin level in the LQZ-7-3 treatment group was dramatically decreased as compared to the vehicle group suggesting LQZ-7-3 may function to inhibit tumor xenograft growth by binding to survivin and promoting its degradation *in vivo* (**Figure 32A**). This finding was further validated by immunofluorescence of tissue sections from each group in which survivin, particularly in the nucleus, was dramatically reduced in the LQZ-7-3 treatment group as compared to the vehicle group (**Figure 32B)**.



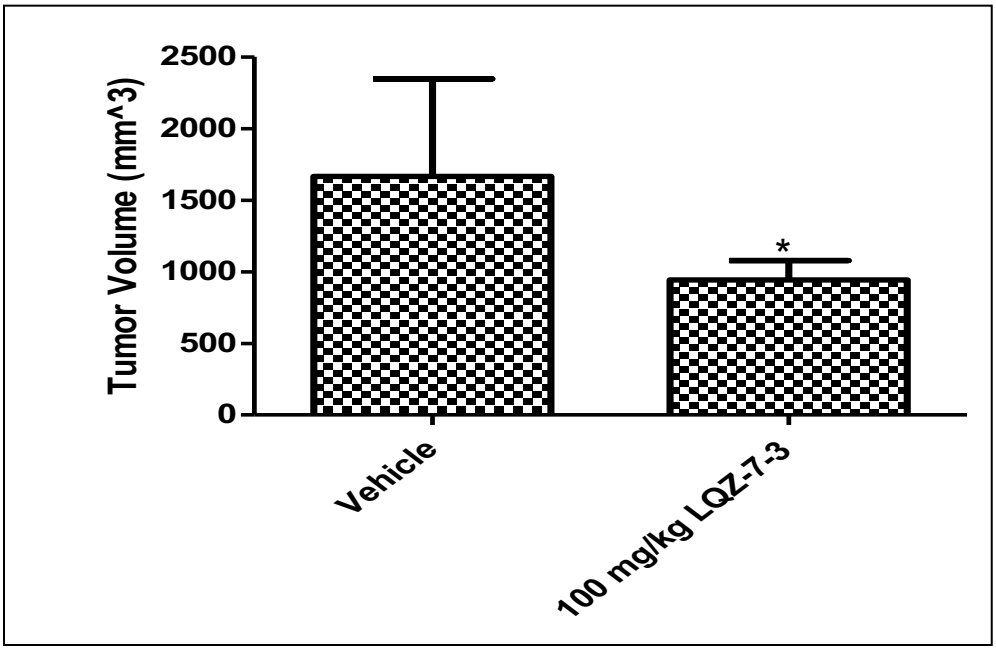


Figure 28. **LQZ-7-3 oral formulation reduces tumor volume.** Tumor volume over the course of the study in which each animal received a total of ten treatments one every other day. The final endpoint had LQZ-7-3 with a significantly lower tumor volume than vehicle treated group. The triangle arrows represent a day in which a treatment was given. * = p-value < 0.05. Error bars equal standard deviation.

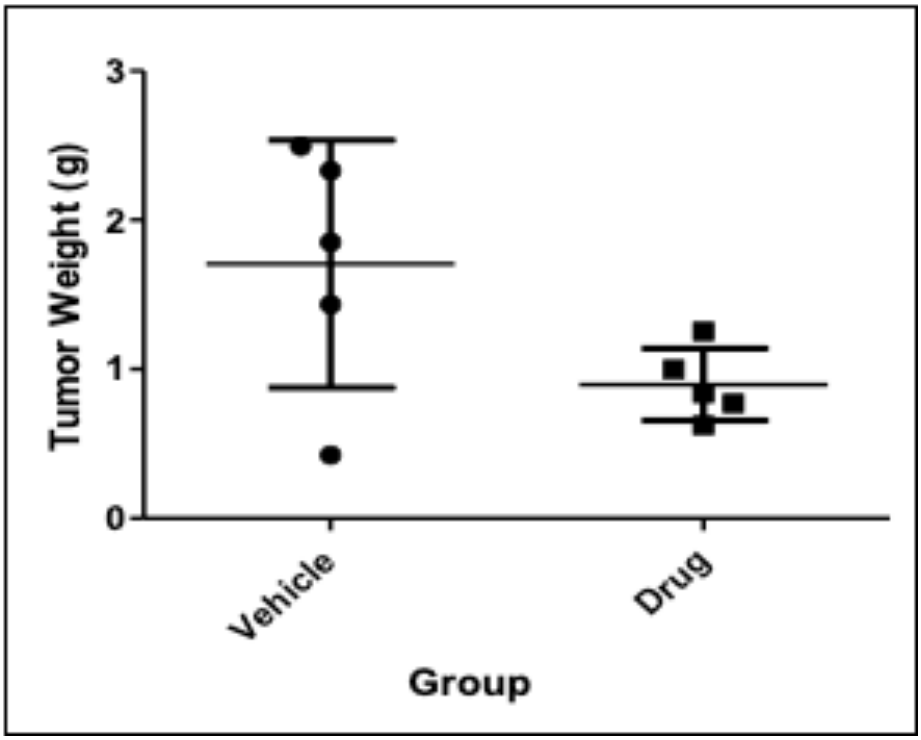


Figure 29. **LQZ-7-3 trends towards a decrease in dry tumor weight.** Dry tumor weight after ten total treatments and conclusion of the study. The final endpoint had LQZ-7-3 treatment group trending towards a significant decrease in tumor dry weight however one mouse tumor in the vehicle group did not graft as well as the others and remained quite small throughout the study.

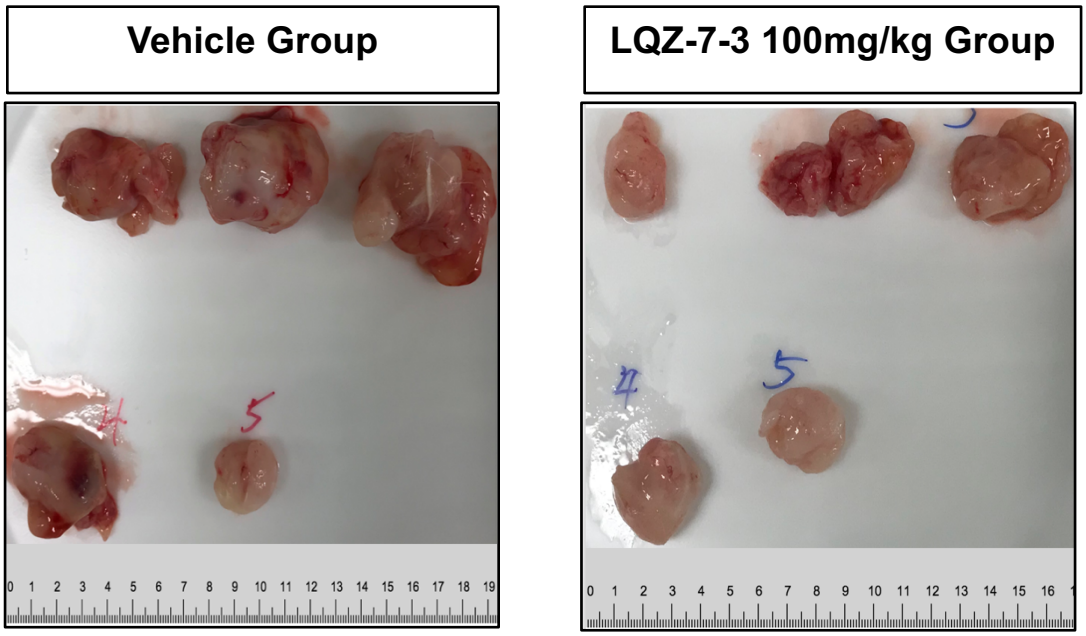
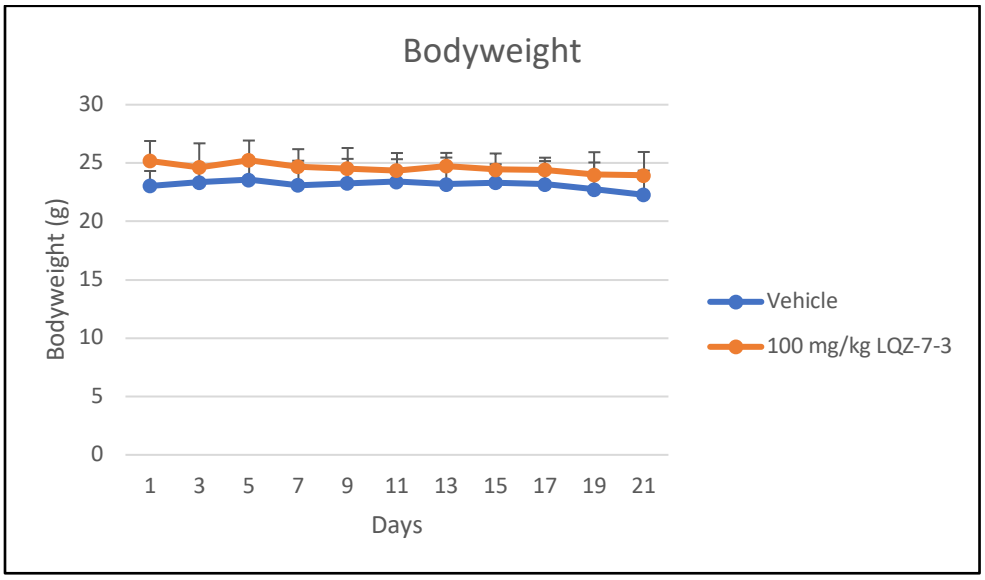


Figure 30. **LQZ-7-3 treatment results in smaller, less aggressive looking tumors.** Tumors in the LQZ-7-3 treatment group appear to be smaller, less red and vascularized as compared to most of the tumors in the vehicle group.

A



B

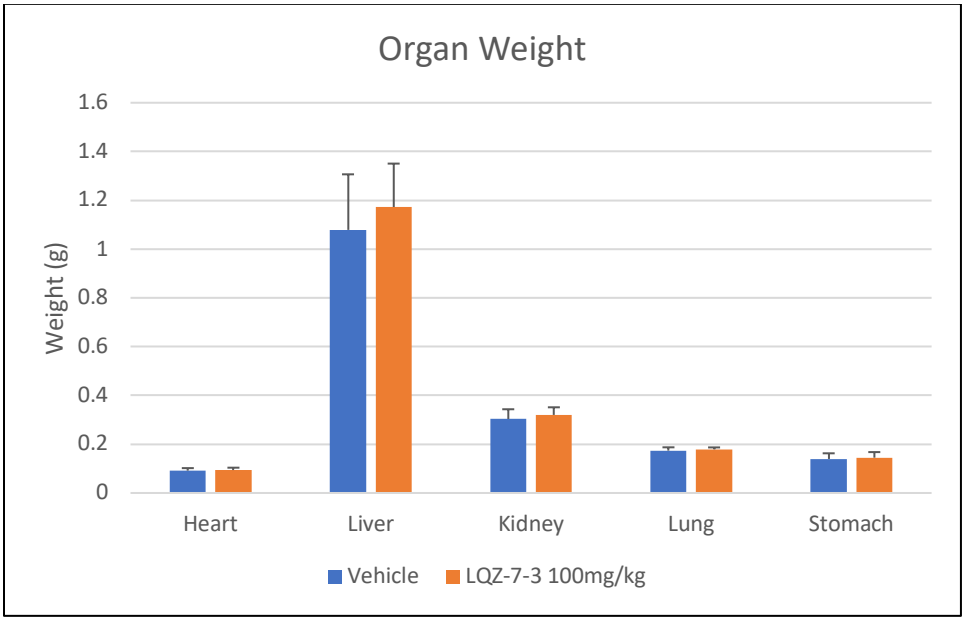


Figure 31. **LQZ-7-3 treatment did not result in observable toxicities over the course of the study.** (A) Bodyweight did not noticeably decrease in either group during the efficacy study. (B) Dry weight of major organs did not show significant differences between the two treatment groups at the time of sacrifice. Error bars indicate standard deviation.

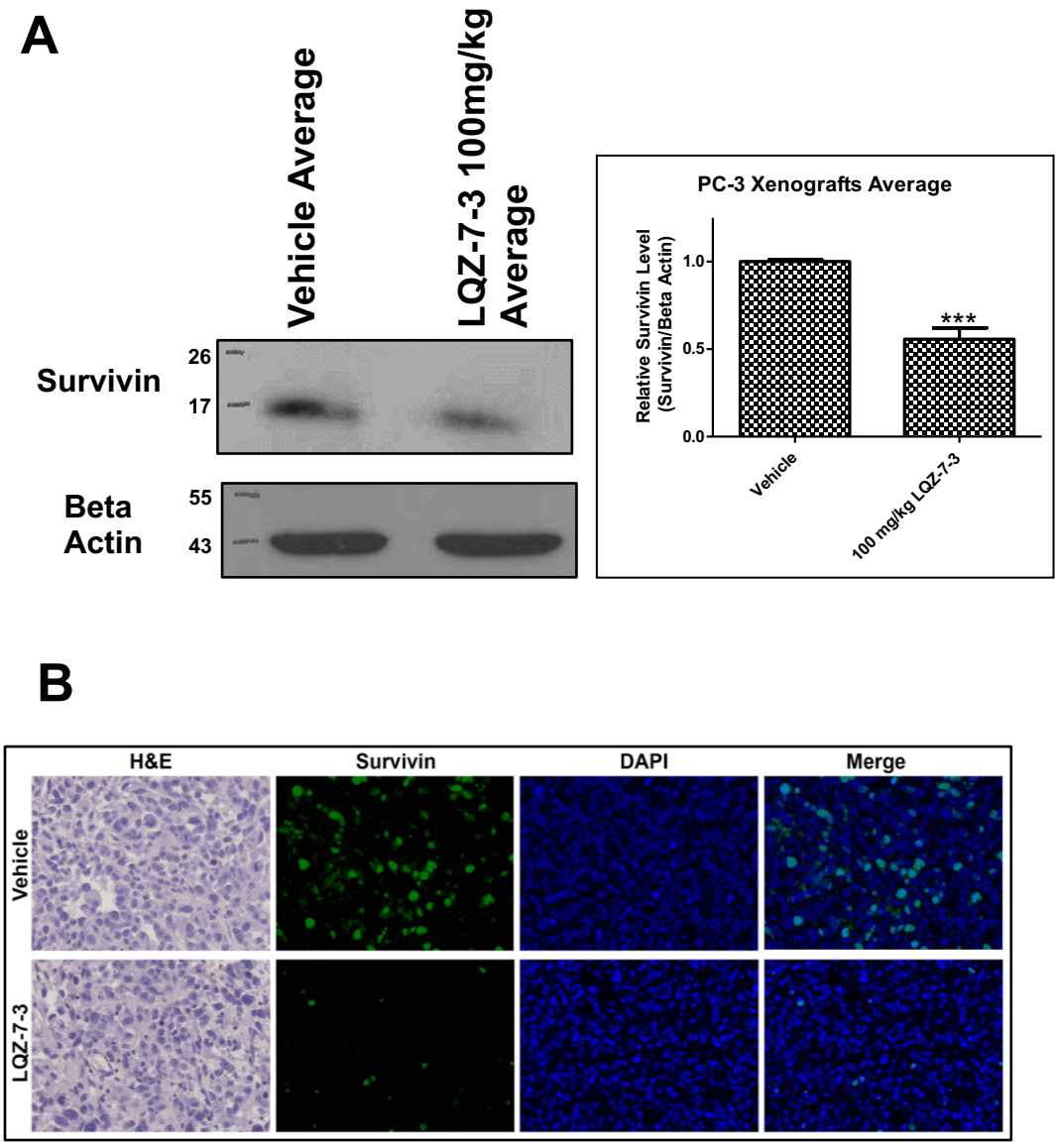


Figure 32. **LQZ-7-3 oral formulation treatment leads to decrease in target protein survivin.** (A) Average survivin level after taking equal parts sample from each tumor in each group and performing western blot analysis. The LQZ-7-3 treatment group showed decrease target protein survivin levels as compared to average vehicle tumor. *** = p-value < 0.001. n = 3 experimental repeats. Error bars indicate standard deviation. (B) Hematoxylin and eosin staining confirming tumor tissue and immunofluorescence staining demonstrating survivin decrease in tumors treated with LQZ-7-3, particularly in the nucleus. Staining was kindly performed by the Histology Core at The University of Toledo.

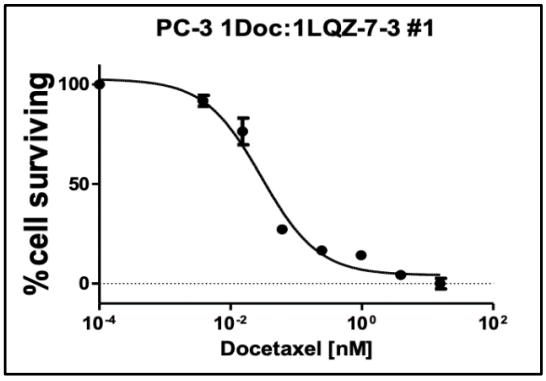
4.2.13 Combination Studies with Docetaxel

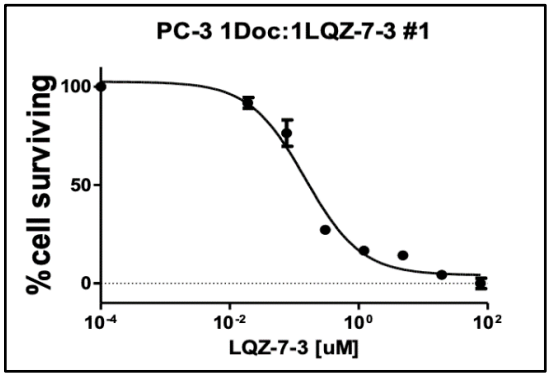
Although LQZ-7-3 was able to reduce tumor volume in a xenograft efficacy study after administration of an oral formulation, tumors were not entirely ablated in the treatment group. As it was the first time performing an efficacy study with one of the survivin inhibitors orally, there is likely dosing and formulation adjustments necessary to make the compound more successful in eliminating tumors in these animals. However, it is also possible that LQZ-7-3 may be more successful in a combinational therapy with more traditional treatments such as docetaxel. In order to assess the potential of administering LQZ-7-3 and docetaxel in a combination therapy *in vivo*, potential synergism between the two agents was first tested in cell based assays.

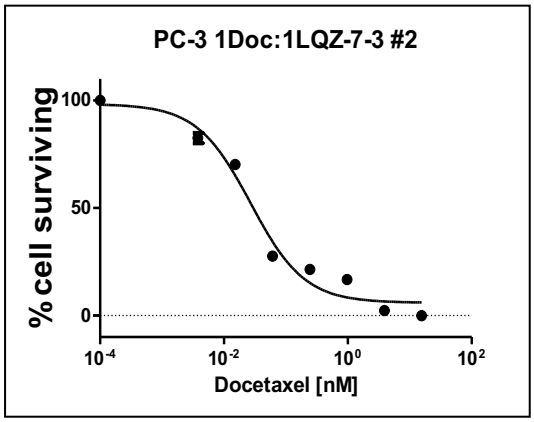
For the cell based assays the CI-isobol method was used to generate a combination index (CI) estimated from the known IC_{50} data of single drug treatments and then dose required to produce the same effect in combination treatments. The CI formula that was utilized is as follows: $CI = C_{A,50}/IC_{50,A} + C_{B,50}/IC_{50,B}$ where $IC_{50,A}$ and $IC_{50,B}$ are the concentration of drug A and drug B that result in 50% inhibition in single drug treatments. $C_{A,50}$ and $C_{B,50}$ represent the concentration of drug a and drug b that provide the same effect in the combination treatment respectively. In this formula a CI value less than one indicates synergism, CI value equal to 1 is additive, and a CI over 1 is antagonism [210]. The results utilizing this method for LQZ-7-3 and docetaxel when given in a combination treatment at a 1:1 ratio of the IC_{50} to IC_{50} indicated strong synergism in both C4-2 and PC-3 cells with CI calculated well under 1

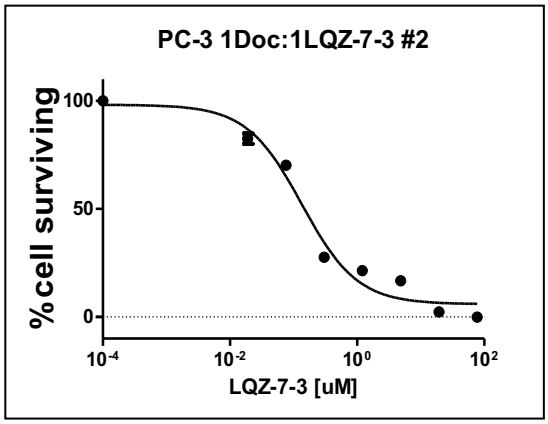
(**Figure 33**). This data provides support that a combination therapy between LQZ-7-3 and docetaxel may be beneficial and synergize in prostate cancer *in vivo* models.

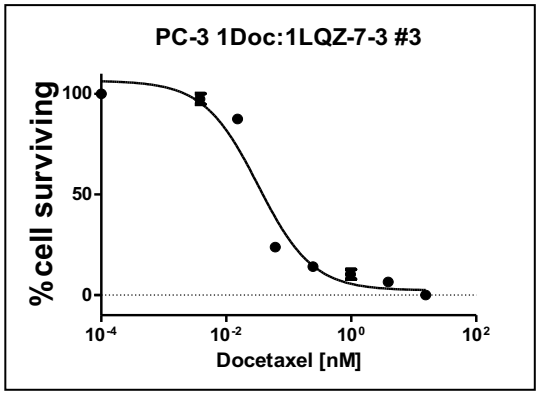
A

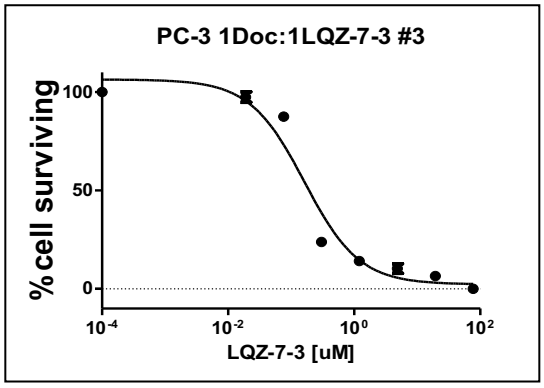

PC-3 1Doc:1LQZ-7-3 #1
% cell surviving
100
50
0
10^{-4}
10^{-2}
10^{0}
10^{2}
Docetaxel [nM]


PC-3 1Doc:1LQZ-7-3 #1
% cell surviving
100
50
0
10^{-4}
10^{-2}
10^{0}
10^{2}
LQZ-7-3 [uM]

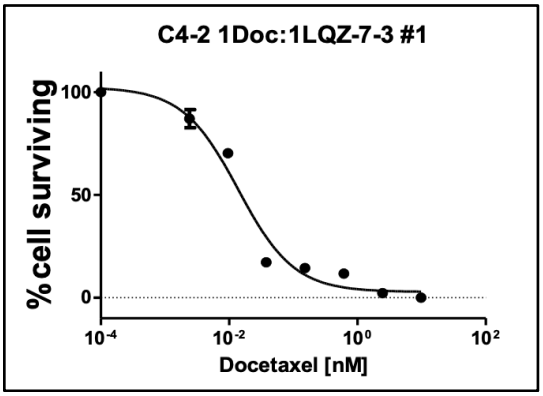

PC-3 1Doc:1LQZ-7-3 #2
% cell surviving
100
50
0
10^{-4}
10^{-2}
10^{0}
10^{2}
Docetaxel [nM]

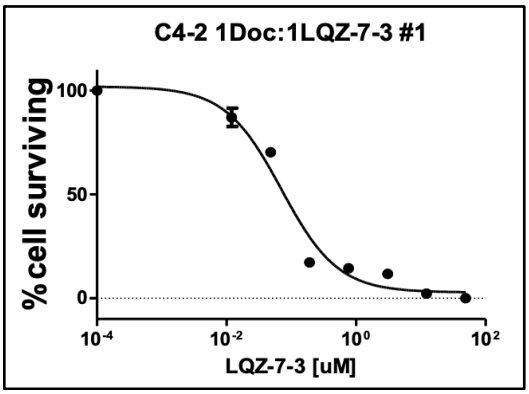

PC-3 1Doc:1LQZ-7-3 #2
% cell surviving
100
50
0
10^{-4}
10^{-2}
10^{0}
10^{2}
LQZ-7-3 [uM]

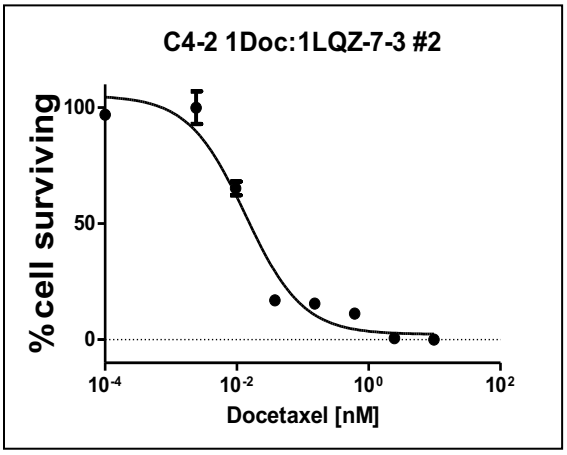

PC-3 1Doc:1LQZ-7-3 #3
% cell surviving
100
50
0
10^{-4}
10^{-2}
10^{0}
10^{2}
Docetaxel [nM]

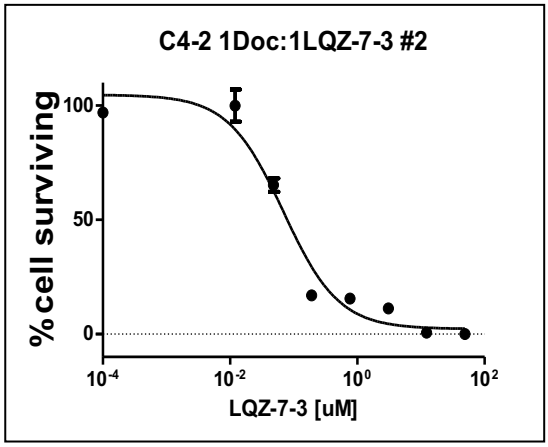

PC-3 1Doc:1LQZ-7-3 #3
% cell surviving
100
50
0
10^{-4}
10^{-2}
10^{0}
10^{2}
LQZ-7-3 [uM]

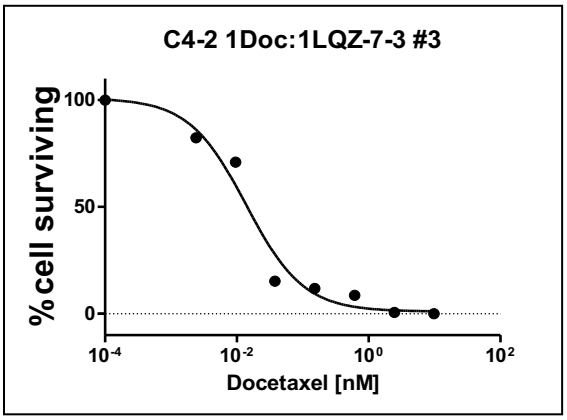
B

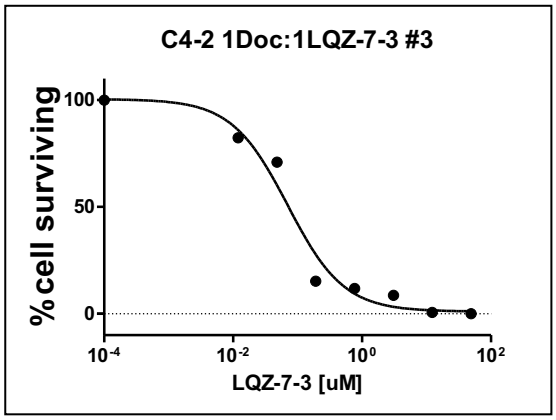

C4-2 1Doc:1LQZ-7-3 #1
% cell surviving
100
50
0
10^{-4}
10^{-2}
10^{0}
10^{2}
Docetaxel [nM]


C4-2 1Doc:1LQZ-7-3 #1
% cell surviving
100
50
0
10^{-4}
10^{-2}
10^{0}
10^{2}
LQZ-7-3 [uM]

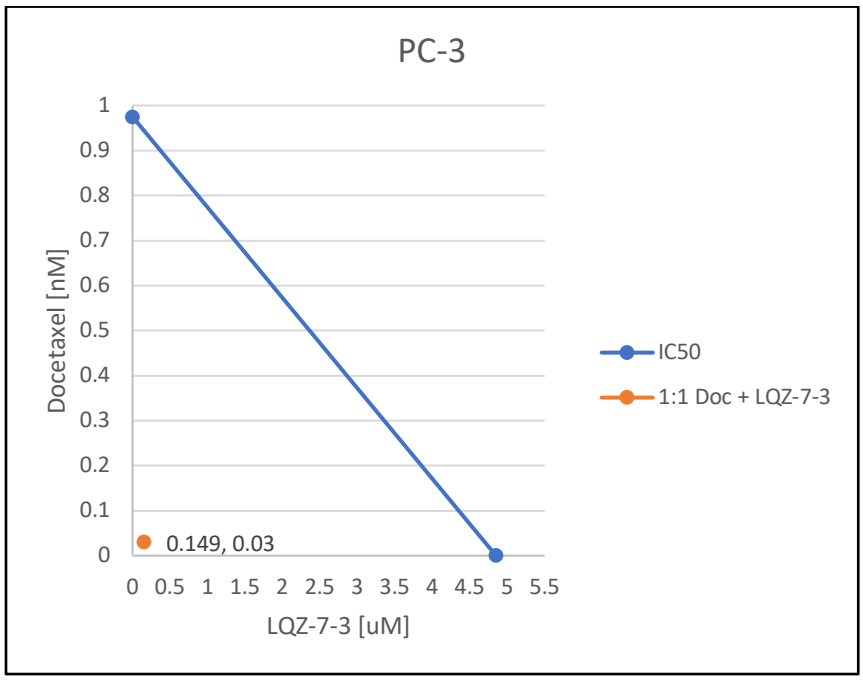

C4-2 1Doc:1LQZ-7-3 #2
% cell surviving
100
50
0
10^{-4}
10^{-2}
10^{0}
10^{2}
Docetaxel [nM]


C4-2 1Doc:1LQZ-7-3 #2
% cell surviving
100
50
0
10^{-4}
10^{-2}
10^{0}
10^{2}
LQZ-7-3 [uM]

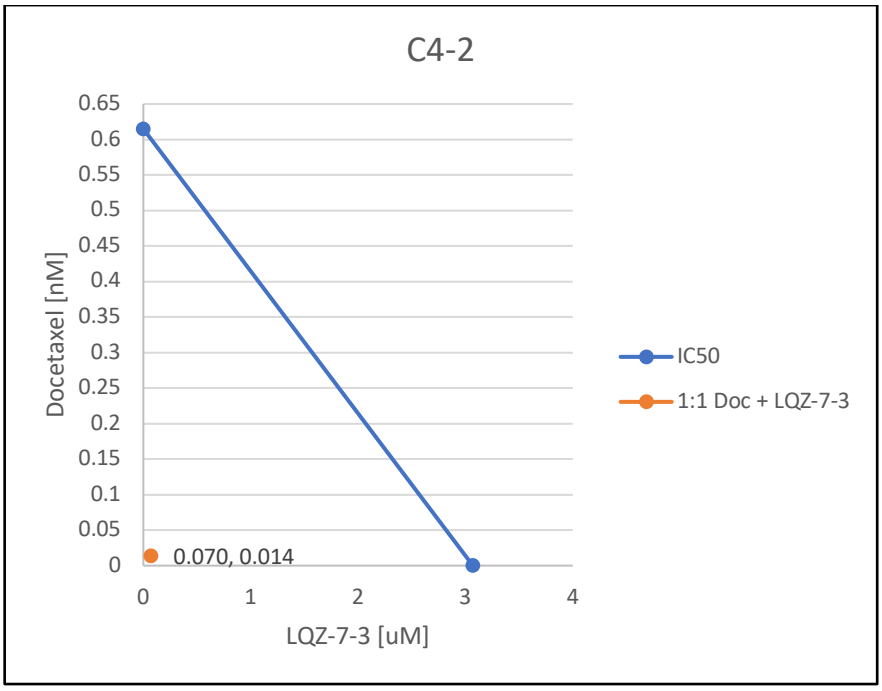

C4-2 1Doc:1LQZ-7-3 #3
% cell surviving
100
50
0
10^{-4}
10^{-2}
10^{0}
10^{2}
Docetaxel [nM]


C4-2 1Doc:1LQZ-7-3 #3
% cell surviving
100
50
0
10^{-4}
10^{-2}
10^{0}
10^{2}
LQZ-7-3 [uM]

C


PC-3
Docetaxel [nM]
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.149, 0.03
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5
LQZ-7-3 [uM]
IC50
1:1 Doc + LQZ-7-3

D


C4-2
Docetaxel [nM]
0.65
0.6
0.55
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0.070, 0.014
0 1 2 3 4
LQZ-7-3 [uM]
IC50
1:1 Doc + LQZ-7-3

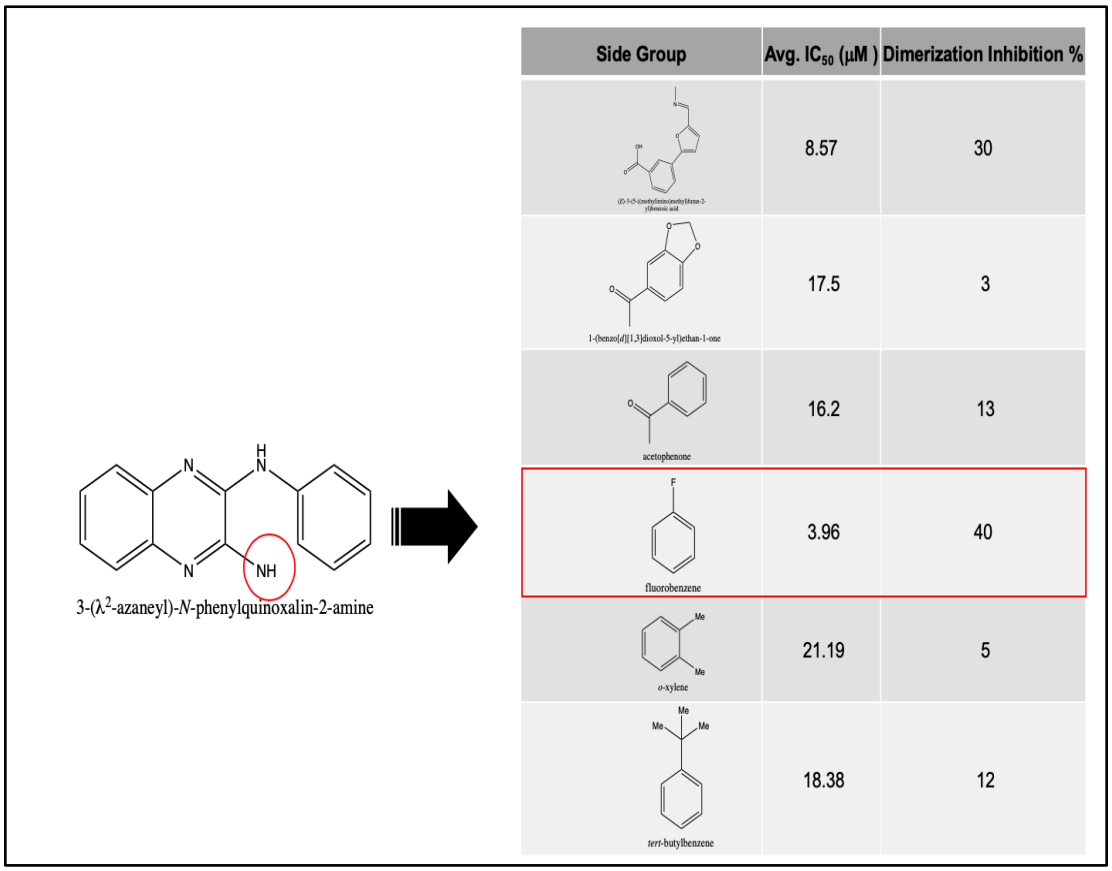
E

Ratio (IC_{50}:IC_{50})	C4-2 CI	PC-3 CI
1 :1	0.046	0.062

Figure 33. **LQZ-7-3 synergizes with docetaxel in vitro.** Representative methylene response curves in combination treatments of LQZ-7-3 and docetaxel for (A) PC-3 cells and (B) C4-2 cells. (C) PC-3 and (D) C4-2 cells Isobologram analysis demonstrating strong synergism between docetaxel and LQZ-7F-1 in. Each point represents the average of three independent experiments. (E) Summarized CI value for both cell lines.

4.2.14 LQZ-7 Analogues Functional Group Evaluation

The way in which the design and synthesis of the LQZ-7 structural analogues was performed has allowed for information to be garnered from the effect of different functional group additions to specific areas of the backbone of LQZ-7. Data from the cytotoxic and mammalian two hybrid assays was utilized to specifically assess the effect of additions of different moieties to the amine group in the LQZ-7 backbone. As shown in **Figure 34**, there was clear evidence that the addition of fluorinated benzene to the amine group established a significantly more potent compound in terms of cell cytotoxicity and ability to inhibit dimerization in the two hybrid assay than other analogues. The addition of methyl groups to the benzene ring also significantly decreased potency and ability to inhibit in the dimerization assay compared to fluorine atom alone. There was also no clear difference between bulky moieties like acetophenone, benzo-dioxolyl ethanone, o-xylene, or tert-butyl benzene rings in the assays utilized. Overall, the fluorinated benzene ring of LQZ-7-3 performed better than any other modalities in these experiments.



Side Group	Avg. IC_{50} (μM)	Dimerization Inhibition %
	8.57	30
1-(benzo[d][1,3]dioxol-5-yl)ethan-1-one	17.5	3
acetophenone	16.2	13
fluorobenzene	3.96	40
o-xylene	21.19	5
tert-butylbenzene	18.38	12

Figure 34. **Functional group analysis of analogue changes at the amine group position of LQZ-7.** LQZ-7-3 fluorinated benzene ring had enhanced cytotoxicity in cell lines as well as improved performance in dimerization assay compared to other functional group changes at the same position.

4.3 Concluding Remarks

The data in this section serve as a first generation optimization of the LQZ-7 survivin dimerization inhibitor backbone. In the outcomes above, a structural analogue of LQZ-7 was found that has enhanced cytotoxicity in prostate cancer cells and inhibits survivin dimerization in a mammalian two hybrid assay greater than the parental compound. The compound, LQZ-7-3, has a strong positive correlation between survivin expression and cytotoxicity in prostate cancer cell lines. LQZ-7-3 also appears to preferentially cause survivin degradation over other IAP family members. Survivin degradation after LQZ-7-3 treatment occurs earlier than 24 hours in both PC-3 and C4-2 and LQZ-7-3 significantly reduces the half-life of survivin. The loss of survivin caused by LQZ-7-3 is blocked by pretreatment with proteasome inhibitors suggesting LQZ-7-3 induced survivin degradation is via the proteasome. LQZ-7-3 increases cellular apoptosis of prostate cancer cells compared to control as measured by annexin V staining and increases in cleaved caspase 3 protein levels. In a xenograft efficacy study, LQZ-7-3 reduced tumor volume after administration of ten oral treatments as compared to vehicle control. Importantly, LQZ-7-3 synergizes with docetaxel warranting the exploration of a combination therapy in future xenograft efficacy studies. Finally, the fluorinated benzene ring of LQZ-7-3 confers improved cellular responses in multiple assays as compared to other functional group substitutions at the same position of the LQZ-7 backbone.

CHAPTER 5. LQZ-7F-1 LOCKED SCAFFOLD OPTIMIZATION

5.1 Background and Rationale

In the previous chapter, the discovery of the original hit compound, LQZ-7, and the next iterative generation analogue, LQZ-7-3 with enhanced cytotoxicity was characterized. The goal of this chapter was to examine a different compound with a more locked backbone, LQZ-7F, which was also identified in the same publication as LQZ-7. It was hypothesized that an optimized LQZ-7F inhibitor would have the greatest cytotoxicity in prostate cancer cells. The data in this section, identify a new lead compound, LQZ-7F-1, that has enhanced cellular cytotoxicity and promotes survivin degradation at substantially lower concentrations. The survivin loss resulting from LQZ-7F-1 treatment is also via a proteasome dependent mechanism. LQZ-7F-1 increases cellular apoptosis of prostate cancer cells as measured by flow cytometry and cleaved caspase 3 protein level increases. The positive cellular data resulting from the use of this compound warrants future exploration in a *in vivo* xenograft efficacy study.

5.1.1 Discovery of LQZ-7F

After the discovery of the original primary hit, LQZ-7, several analogues were generated and tested by similar means as before. One such compound, LQZ-7F, displayed more potent inhibition of cancer and had a strong positive correlation between survivin expression and cytotoxicity in these cancer cell lines. More importantly, in an *in vivo* xenograft efficacy model, LQZ-7F treatment via i.p. injection significantly inhibited the growth of prostate tumors growth [61].

The interesting feature of LQZ-7F is that it was the only compound identified in the screenings that had a locked back bone conformation consisting of 2 five-member rings and two-six member rings. The others compounds all consisted of a five member ring and dihydropyrazine ring connected other functional groups by amine groups creating this more flexible compound. The uniqueness of the backbone and preclinical success of this compound made this compound an ideal candidate for additional screening for an optimized structural derivative.

5.2 Results

5.2.1 LQZ-7F Analogue Generation

The first generation of LQZ-F structural analogues were synthesized maintaining the 4 ring backbone of LQZ-7F that is critical for the π-π stacking interactions with the dimerization core of survivin and changing the functional group attached to the cyclopentane ring. The idea behind additions of different functional groups at this position was to find an optimized functional group that strongly interacts with the survivin backbone. The functional group changes consisted of the addition of a simple carbonyl group to the cyclopentane to the addition of a complex n-amino-oxadiazolyl-butyramide group to the same position. The fifteen structural analogues of LQZ-7F are shown in **Figure 35**.

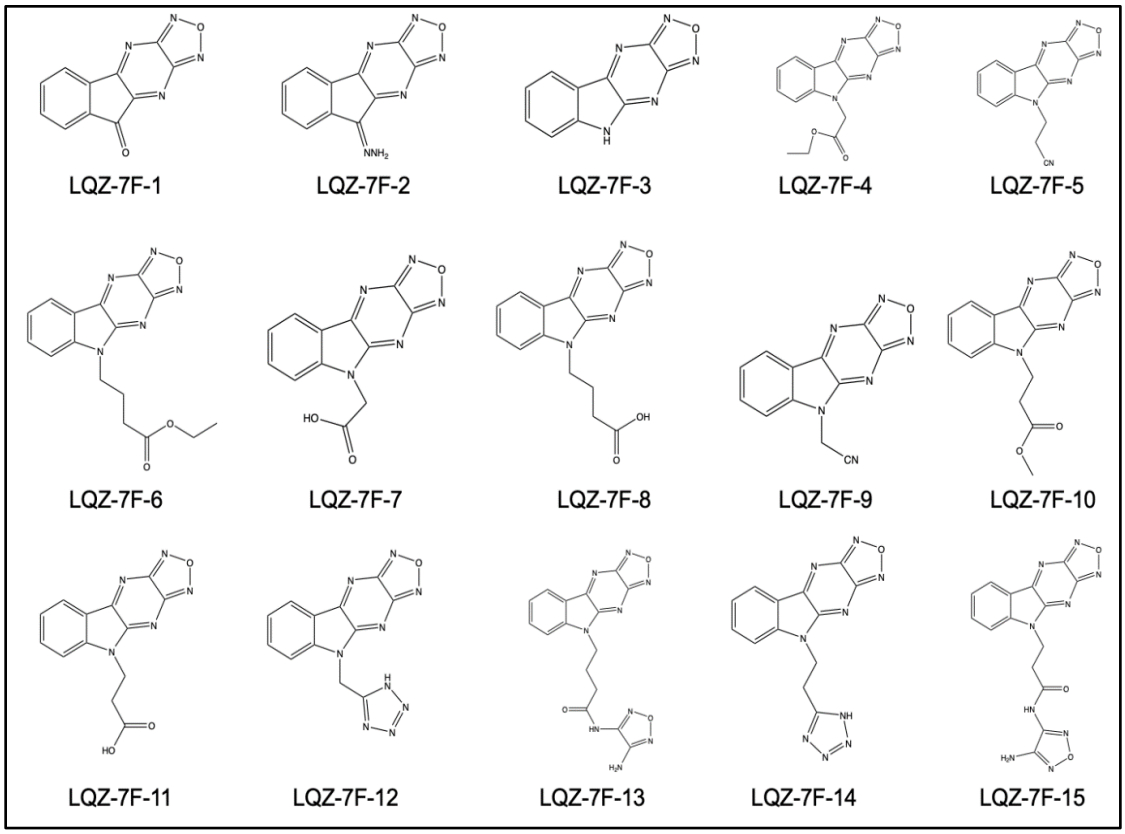


Figure 35. **Chemical structures of LQZ-7F analogues with substitutions at cyclopentane group.**

5.2.2 LQZ-7F Analogues Single Concentration Analysis

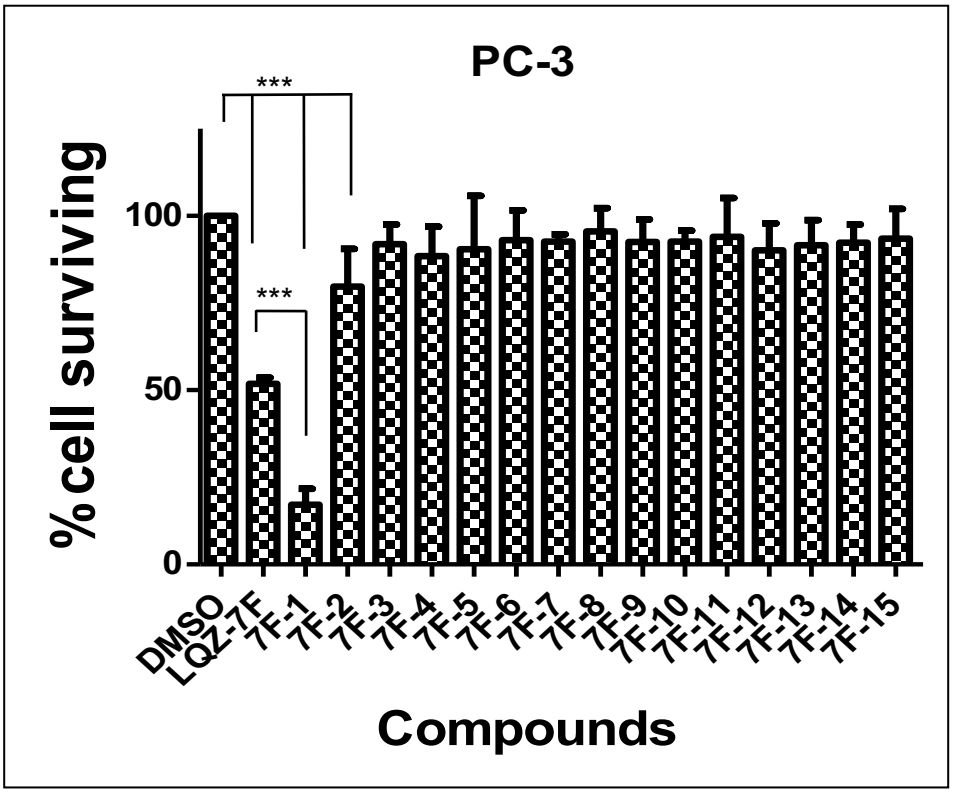
In order to determine if any of the LQZ-7F structural analogues had a greater ability to promote cancer cell killing than LQZ-7F, a single concentration analysis was first performed in PC-3 and C4-2 cells. For this series of experiments, cells were treated with 2.5 μM of each different compound for 72 hours. This concentration was specifically chosen as it represents the average IC_{50} value of LQZ-7F in prostate cancer cells. The results from the single concentration methylene blues are shown in **Figure 36**. As excepted, treatment of LQZ-7F at 2.5 μM inhibited both PC-3 and C4-2 roughly 50%. The only analogue that promoted cell killing to a greater extent than the parental compound was, LQZ-7F-1. This compound with the carbonyl substitution on the cyclopentane had less than 20% cells surviving in both cell lines after 72 hour treatment. The data in this section positioned LQZ-7F-1 for further in vitro testing to determine if may represent a more potent survivin inhibitor than LQZ-7F.

5.2.3 LQZ-7F-1 Cytotoxicity

The results of the single concentration analysis indicated that LQZ-7F-1 is a more potent inhibitor than LQZ-7F. To clearly define the IC_{50} values of LQZ-7F in prostate cancer cells and additional methylene blue experiment was performed. PC-3 and C4-2 were tested with LQZ-7F-1 over a concentration range of 1 nM to 20 μM. As shown in **Figure 37**, LQZ-7F-1 had a significantly lower IC_{50} value than LQZ-7F-1 with IC_{50} values of 158 nM and 170 nM in PC-3 and C4-2 respectively. In fact, LQZ-7F-1 represents the first survivin inhibitor

identified in the screening process to show a sub micromolar IC_{50} in cancer cells in vitro.

A


PC-3
***
***
% cell surviving
100
50
0
DMSO
LQZ-7F
7F-1
7F-2
7F-3
7F-4
7F-5
7F-6
7F-7
7F-8
7F-9
7F-10
7F-11
7F-12
7F-13
7F-14
7F-15
Compounds

B

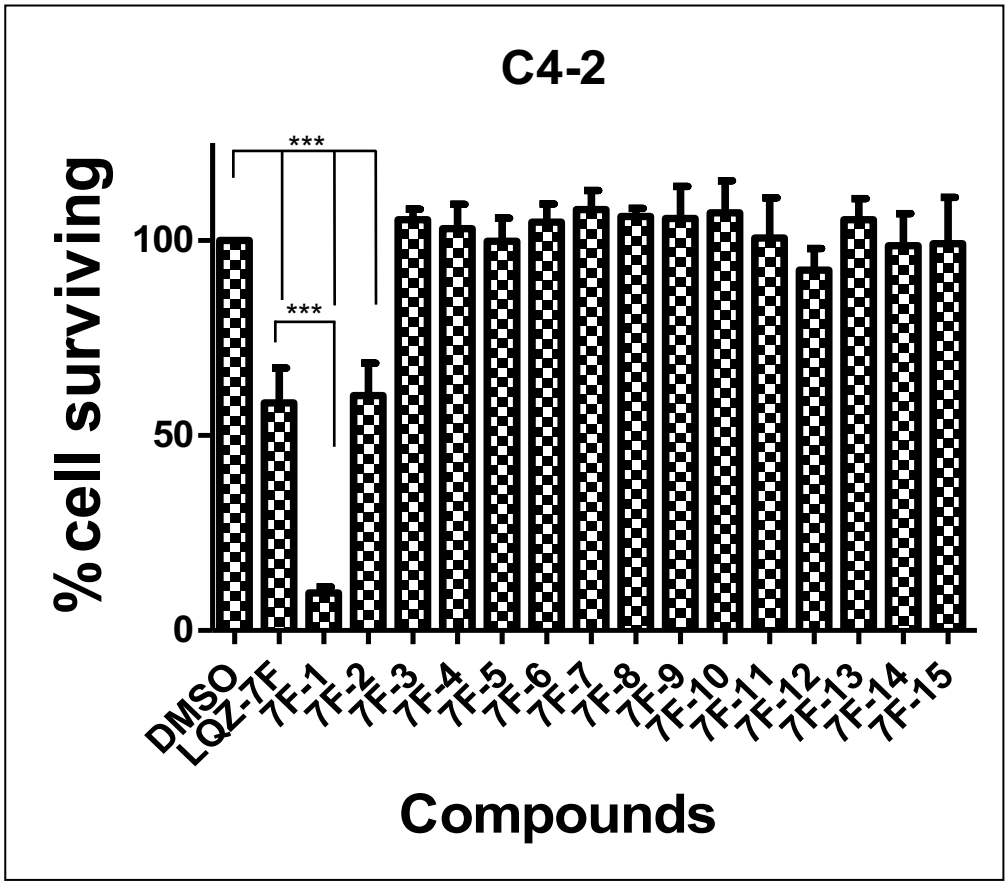
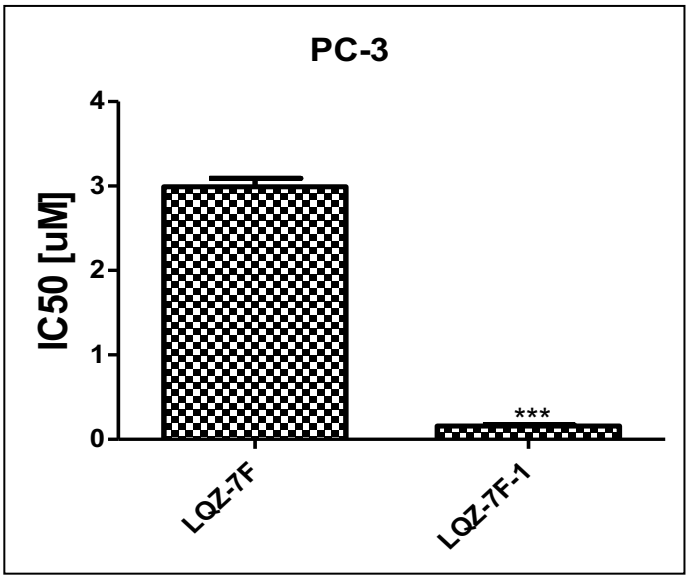


Figure 36. **Single concentration analysis of LQZ-7F and structural analogues.** (A) PC-3 and (B) C4-2 cells were treated with 2.5 μM of each compound for 72 hours. The results indicate the percentage cells surviving after treatment. Only LQZ-7F-1 had a greater amount of cell killing as compared to parental LQZ-7F. Each data point was performed in triplicate. *** = p-value <0.001. n = 3 independent experiments. Error bars equal standard deviation.

A



B

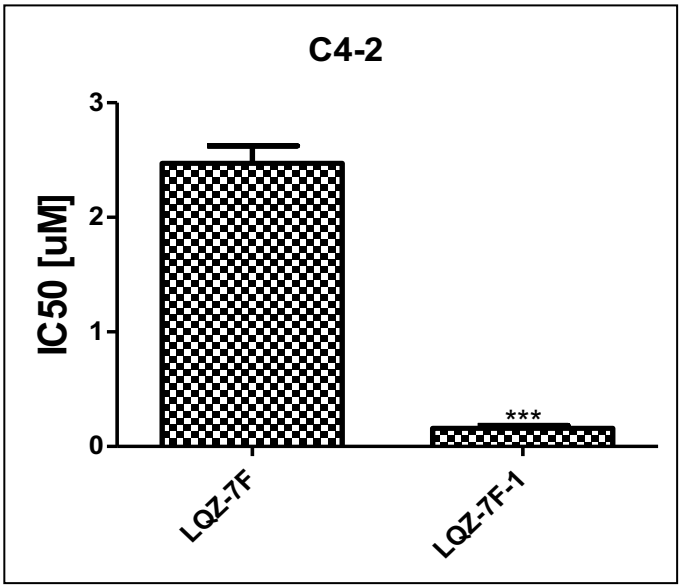


Figure 37. **LQZ-7F-1 is more potent inhibitor than LQZ-7F in prostate cancer cells.** LQZ-7F-1 has a significantly lower IC_{50} value in (A) PC-3 (158 nM) and (B) C4-2 (175 nM) cells than parental compound LQZ-7F. LQZ-7F-1 is the first sub micromolar inhibitor identified during screening. Each concentration was performed in triplicate. *** = p-value < 0.001. n = 3 independent experiments. Error bars equal standard deviation.

5.2.4 Mammalian Two Hybrid LQZ-7F-1

After confirming the increase in potency by dose response curves, the next step was to establish that LQZ-7F-1 also exerts its function through disruption of survivin dimerization as expected. Similarly to the previous section, a mammalian two hybrid assay was performed to compare the dimerization inhibition by LQZ-7F and LQZ-7F-1 where group 1 represents the cloned survivin plasmids and group 4 are empty vectors controls for measuring basal RLU (SEAP) levels in the media. Treatment with LQZ-7F and LQZ-7F-1 both significantly reduced survivin dimerization in this assay (**Figure 38**). However, LQZ-7F-1 decreased SEAP reporter gene production in survivin containing plasmid group to a greater extent than LQZ-7F. Importantly, neither compound interfered with the empty vector controls relative light values. The data in this section indicates LQZ-7F-1 is not only a more potent inhibitor in terms of cell killing but also interfering with the intended target survivin's dimerization.

5.2.5 LQZ-7F-1 Cytotoxicity in Survivin Overexpression Cells

To determine if survivin overexpressing cells have a decreased sensitivity to LQZ-7F-1, C4-2 survivin stable overexpressing cells were once again used in a cell cytotoxicity assay. After 72 hour treatment, the C4-2 vector cells had an IC_{50} of roughly 200 nM, while C4-2 survivin cells had a significantly higher IC_{50} of 947 nM (**Figure 39**). The data indicate that overexpression of survivin decreases sensitivity to LQZ-7F-1 as expected.

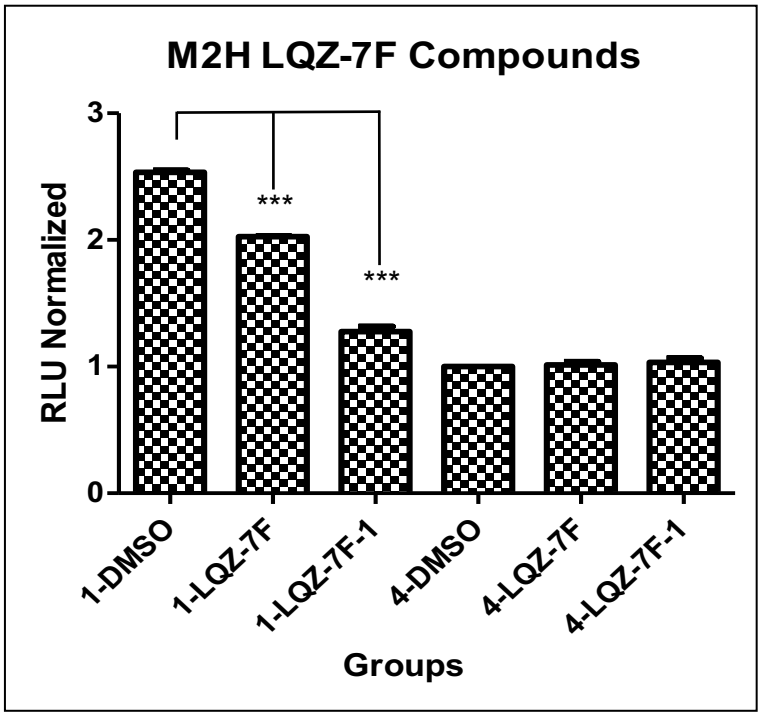


Figure 38. **LQZ-7F-1 decreases survivin dimerization greater than LQZ-7F in a mammalian two hybrid assay.** LQZ-7F-1 had a significantly lower normalized RLU value than LQZ-7F, indicating a decreases in survivin dimerization in the assay. Each compound was utilized at a concentration of 1 μM. Each transfection was performed in triplicate. *** = p-value <0.001. n = 3 independent experiments. Error bars equal standard deviation.

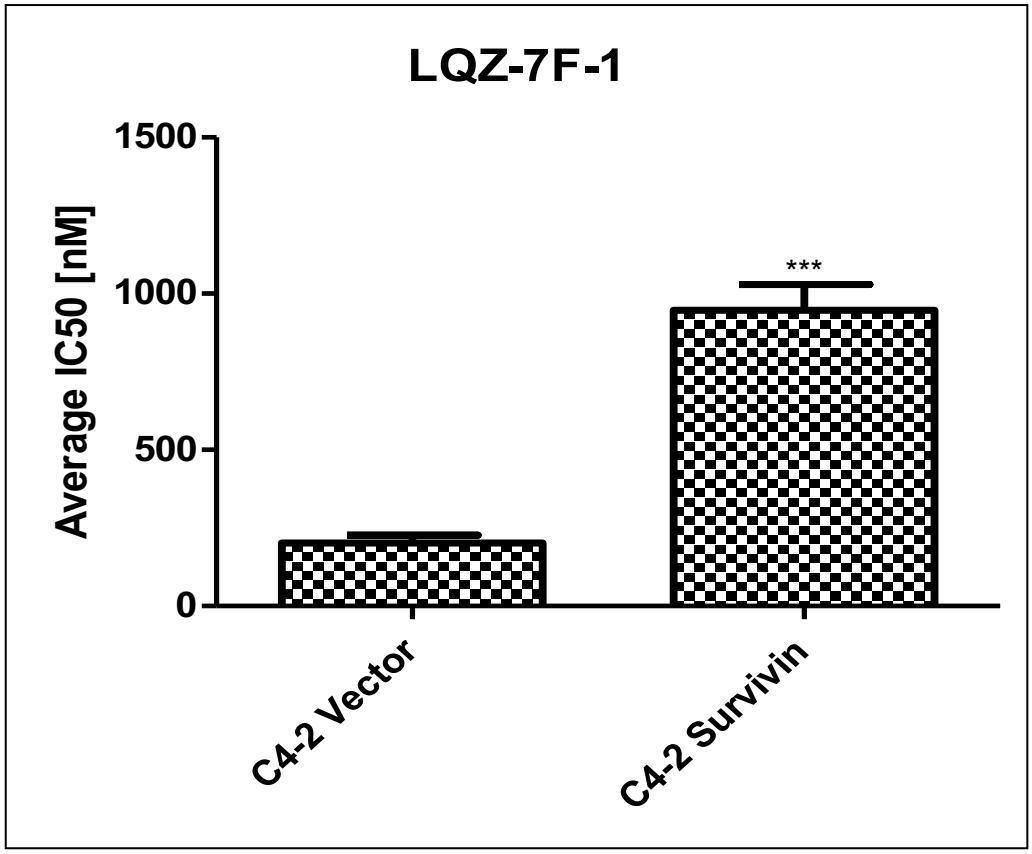


Figure 39. **Survivin overexpression decreases sensitivity to LQZ-7F-1.** LQZ-7F-1 has a significantly higher IC_{50} value in survivin stable overexpression cells. As the intended target of LQZ-7F-1, one would expect increasing survivin levels would require more compound to overcome this increase. *** = p-value < 0.001. n = 3 independent experiments. Error bars equal standard deviation.

5.2.6 LQZ-7F-1 Survivin Degradation

Previous sections have illustrated that this group of survivin inhibitors upon treatment result in prompt survivin loss and degradation in the cell via interference with survivin dimerization interface. To confirm that LQZ-7F-1 also reduces target protein survivin levels in prostate cancer cells, a series of western blot analyses were performed. In PC-3 cells, both LQZ-7F and LQZ-7F-1 were treated at 1 μM for only 8 hours. LQZ-7F-1 robustly decreased survivin levels even as early as 8 hours in these cells (**Figure 40**). LQZ-7F-1 analogue also significantly decreases survivin levels greater than parental compound at this concentration and timepoint. In C4-2 cells, LQZ-7F was treated at 10 μM while LQZ-7F-1 concentration for treatment remained at 1 μM for 8 hours. As shown in **Figure 41**, LQZ-7F-1 even at a lower concentration significantly decreased survivin protein level greater than a higher concentration of LQZ-7F at an 8 hour timepoint. The data indicate LQZ-7F-1 is not only a more inhibitor but also causes survivin loss faster than the progenitor compound, LQZ-7F.

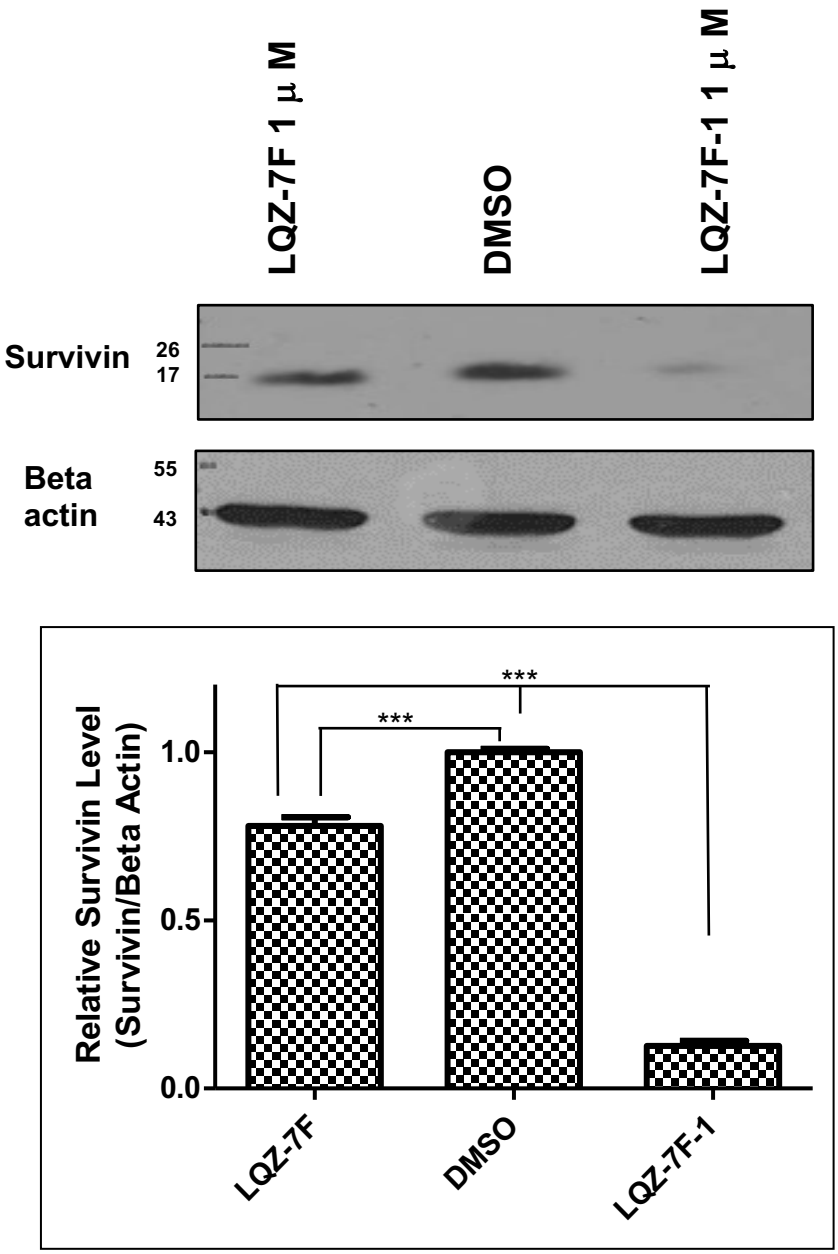


Figure 40. **LQZ-7F-1 decreases survivin protein level greater than LQZ-7F**. LQZ-7F-1 decreases survivin level significantly as early as 8 hours. This decreases is greater than that seen by parental compound LQZ-7F. This experiment was performed in PC-3 cells. *** = p-value < 0.001. n = 3 independent experiments. Error bar equals standard deviation.

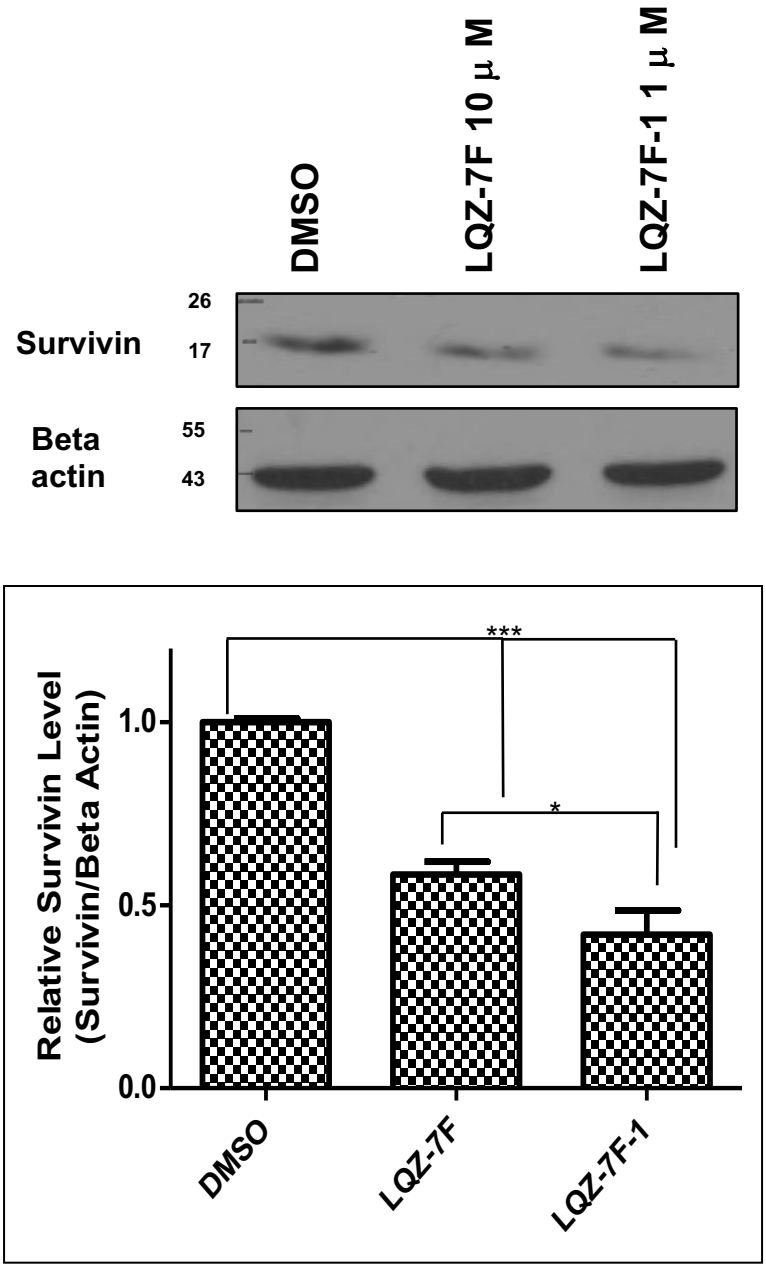


Figure 41. **LQZ-7F-1 decreases survivin protein level greater than previous generation inhibitor even at a significantly lower concentration**. LQZ-7F-1 treated at 1 μM decreases survivin level significantly more than LQZ-7F at 10 μM. This experiment was performed in C4-2 cells. *** = p-value < 0.001. * = p-value < 0.05. n = 3 independent experiments. Error bar equals standard deviation.

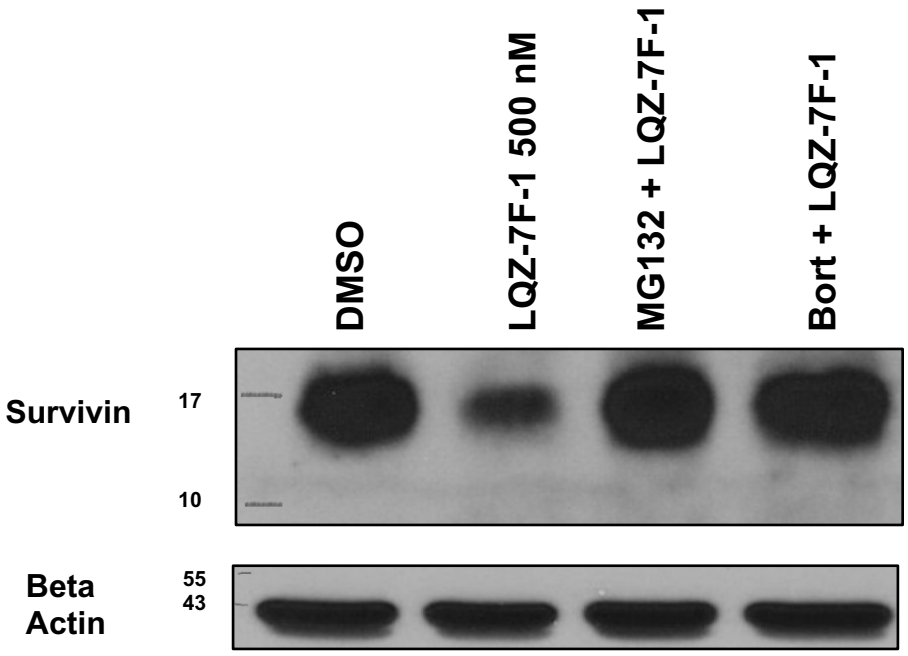
5.2.7 LQZ-7F-1 and Proteasome Inhibitors Experiment

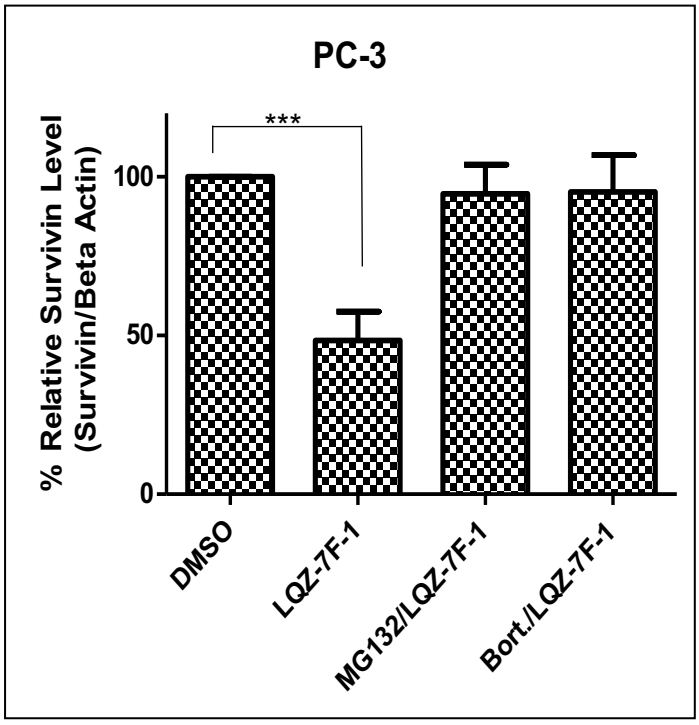
To determine if LQZ-7F-1 also promotes survivin degradation via proteasome, PC-3 and C4-2 cells were pretreated with two different proteasome inhibitors MG132 and bortezomib for two hours before treatment with the compound. As expected LQZ-7F-1 at 500 nM reduced survivin level significantly as compared to DMSO control (**Figure 42A and 42B**). Interestingly, pretreatment with the proteasome inhibitors rescued the survivin level to a similar level as seen in the DMSO treatment group. Thus, it appears LQZ-7F-1 treatment also promotes survivin degradation via the proteasome.

5.2.8 Apoptosis Studies

To determine if LQZ-7F-1 also induces apoptosis of cancer cells Flow Cytometry with Annexin V staining was performed in coordination with the Flow Cytometry Core. Treatment with 200 nM LQZ-7F-1 generated 21.15 and 17.91 relative fold apoptosis increase in PC-3 and C4-2 cells respectively (**Figure 43A and 43B)**. In order to validate the apoptosis data from the annexin v staining, western blotting analysis was performed utilizing an apoptosis marker, cleaved caspase 3, which is activated and protein levels increase during the apoptosis cascade. As shown in **Figure 44A and 44B**, treatment with LQZ-7F-1 also caused an increase in cleaved caspase 3 levels in both PC-3 and C4-2 cells. Overall this section provides evidence that LQZ-7F-1 also promotes cancer cell death after survivin degradation by spontaneous apoptosis.

A


DMSO
LQZ-7F-1 500 nM
MG132 + LQZ-7F-1
Bort + LQZ-7F-1
Survivin
17
10
Beta Actin
55
43


PC-3
% Relative Survivin Level (Survivin/Beta Actin)
***
100
50
0
DMSO
LQZ-7F-1
MG132/LQZ-7F-1
Bort./LQZ-7F-1

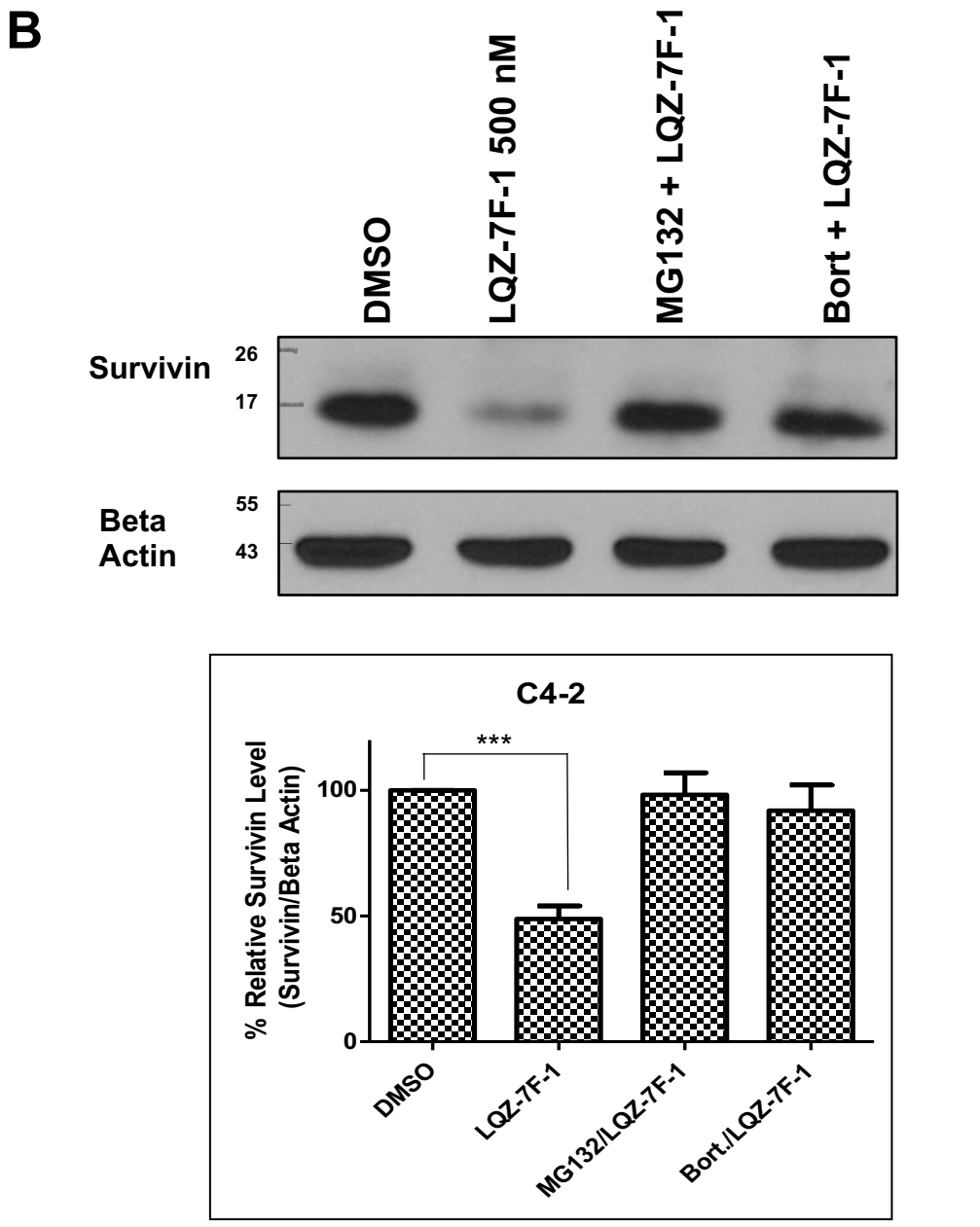


Figure 42. **Pretreatment with proteasome inhibitors rescues LQZ-7F-1 induced survivin degradation.** Survivin loss caused by 24 hour treatment with LQZ-7F-1 was able to be rescued by blocking proteasome activity via pretreatment with two different inhibitors 7 μmol/L MG132, or 70 nmol/L bortezomib for 2 hours in (A) PC-3 and (B) C4-2 cells. Survivin level was nearly completely rescued to DMSO control level. *** = p-value < 0.001. n = 3 independent experiments. Error bar equals standard deviation.

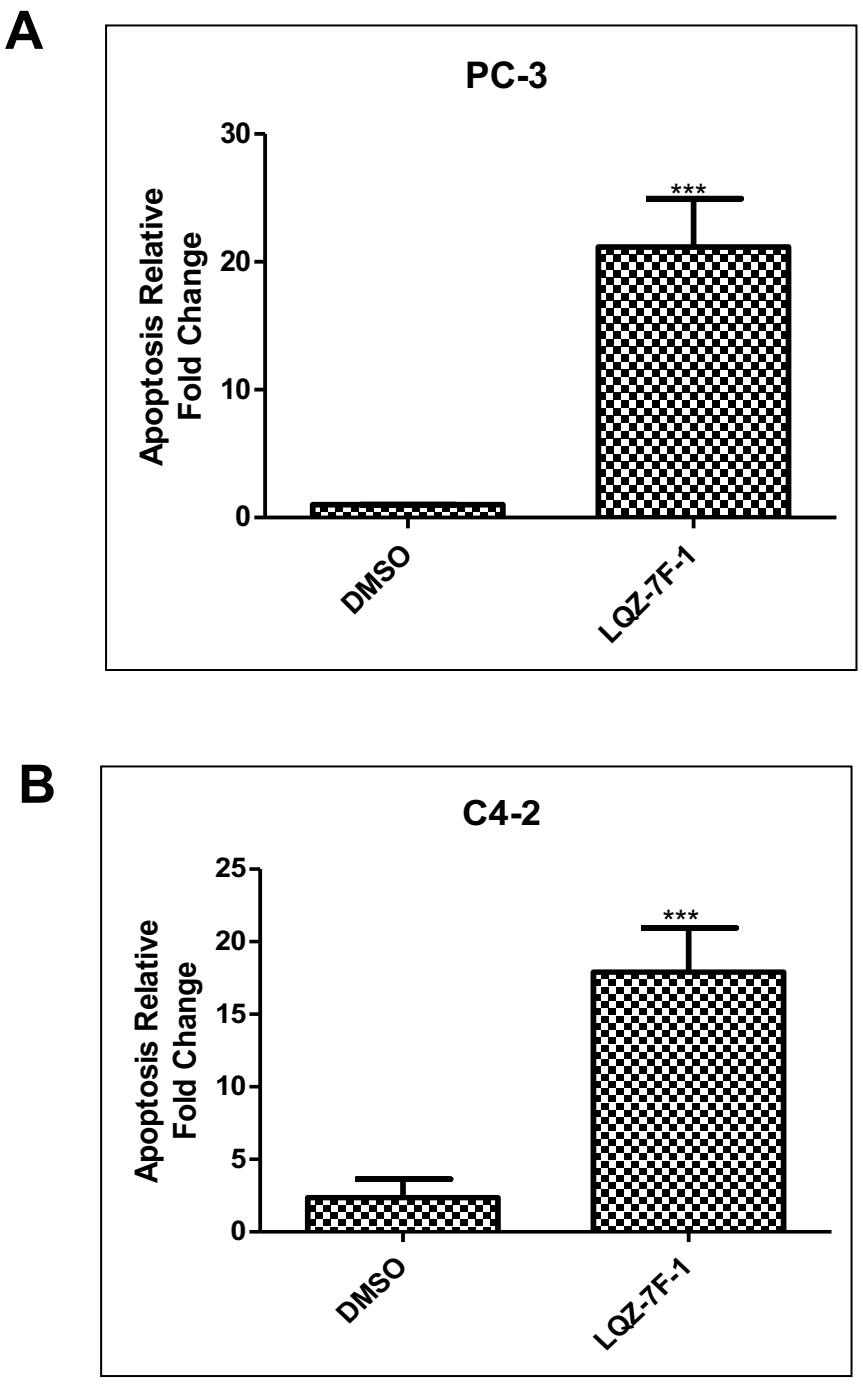
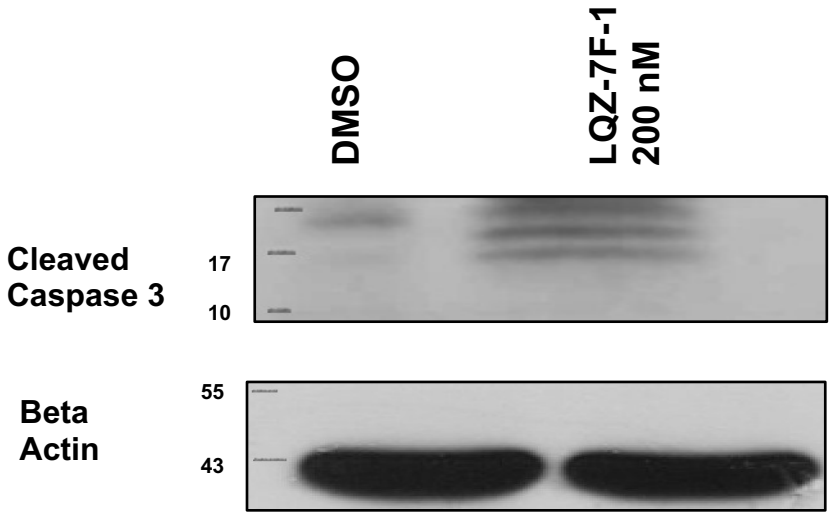
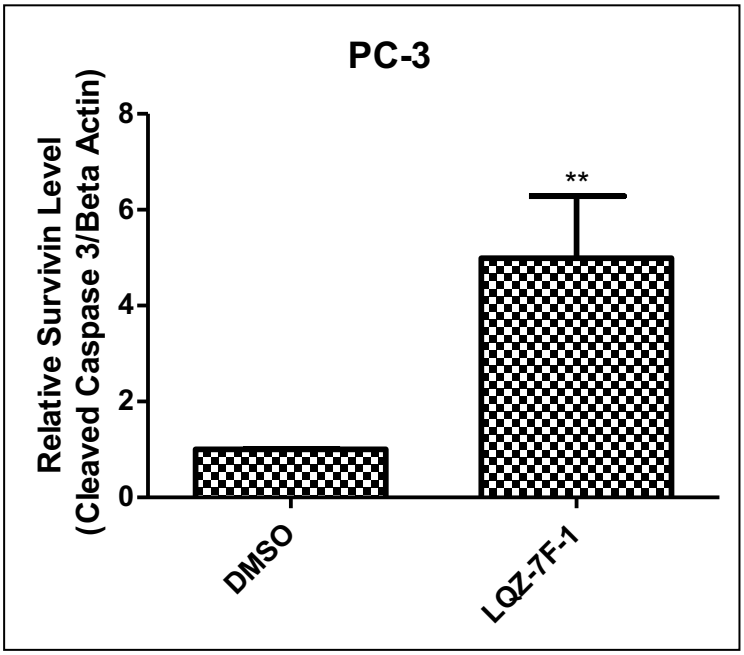


Figure 43. **LQZ-7F-1 treatment causes increased apoptosis in prostate cancer cells.** Survivin loss caused by treatment with LQZ-7F-1 causes increased levels of apoptosis (A) PC-3 and (B) C4-2 cells as evidenced by increased Annexin V staining Flow Cytometry. *** = p-value < 0.001. n = 3 independent experiments. Error bar equals standard deviation.

A


DMSO
LQZ-7F-1
200 nM
Cleaved
Caspase 3
17
10
Beta
Actin
55
43


PC-3
Relative Survivin Level
(Cleaved Caspase 3/Beta Actin)
8
6
4
2
0
**
DMSO
LQZ-7F-1

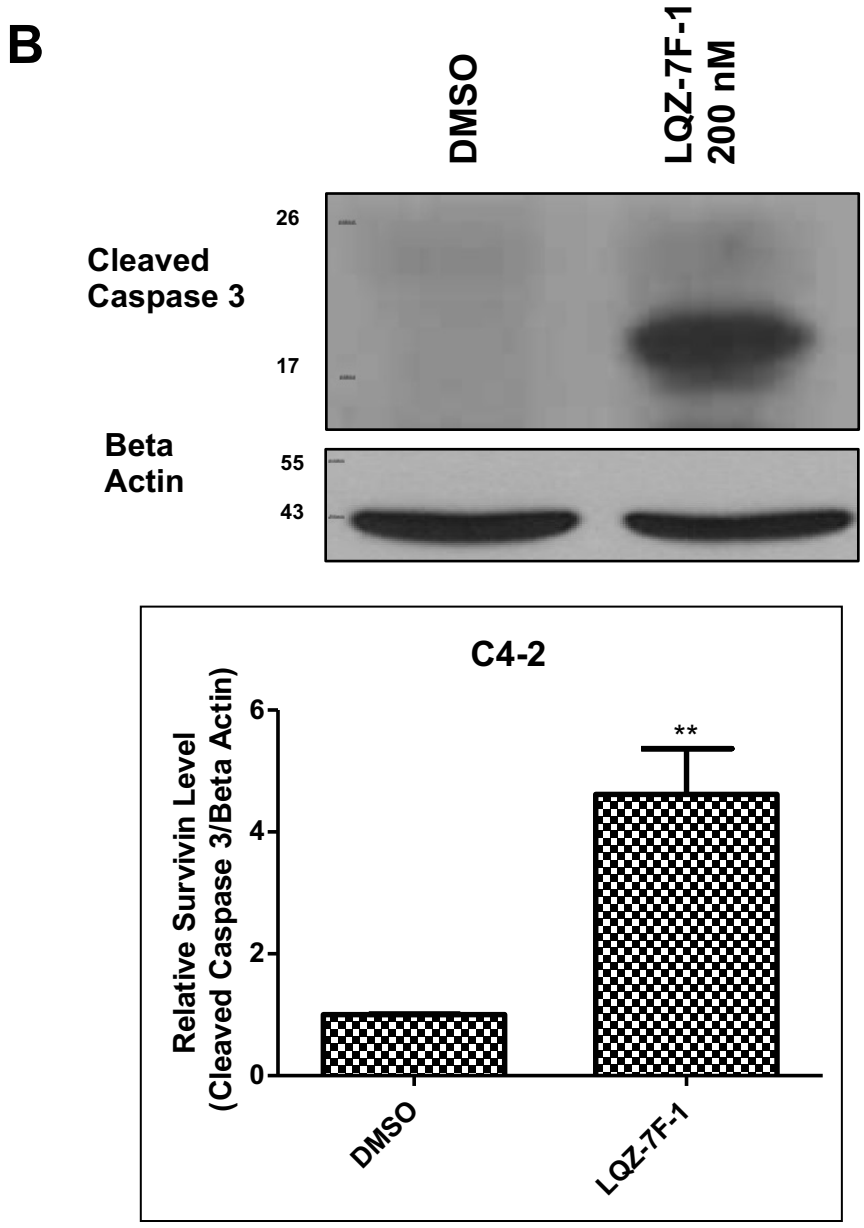
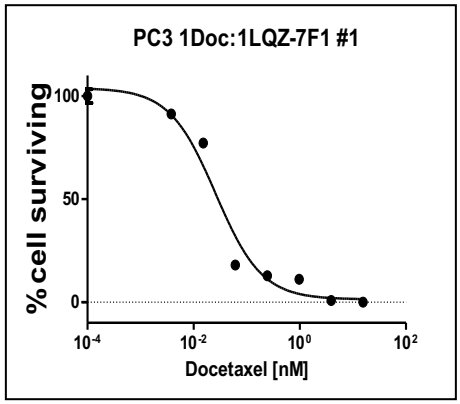


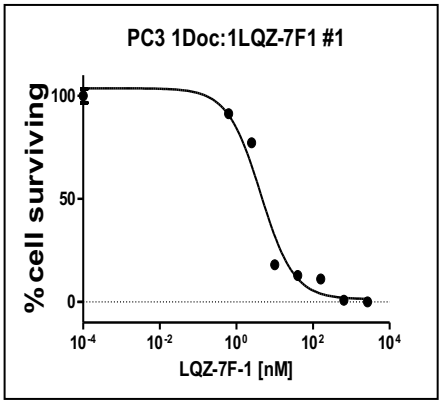
Figure 44. **LQZ-7F-1 treatment causes increased cleaved caspase 3 in prostate cancer cells.** Treatment with LQZ-7F-1 leads to an increase in cleaved caspase 3 levels as seen by western blot analysis in (A) PC-3 and (B) C4-2 cells. The increase in cleaved caspase 3 is confirmation of the Flow cytometry data indicating increased apoptosis after LQZ-7F-1 treatment. ** = p-value < 0.01. n = 3 independent experiments. Error bar equals standard deviation.

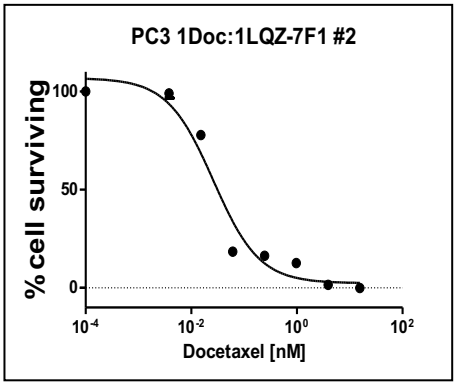
5.2.9 Combination Studies with Docetaxel

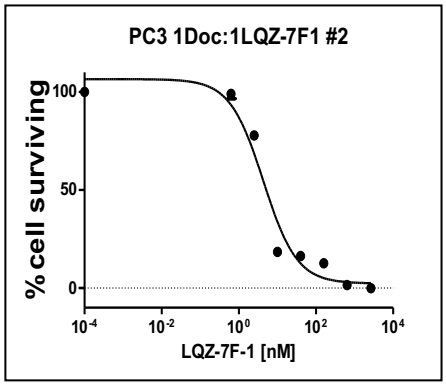
As a future *in vivo* efficacy study utilizing LQZ-7F-1 alone and in combination with docetaxel is likely warranted due to the positive in vitro data, potential synergism between the two agents was first tested in cell based assays. For the cell based assays the CI-isobol method was once again used to generate a combination index (CI) estimated from the known IC_{50} data of single drug treatments and then dose required to produce the same effect in combination treatments. The results utilizing this method for LQZ-7F-1 and docetaxel when given in a combination treatment at a 1:1 ratio of the IC_{50} to IC_{50} indicated strong synergism in both C4-2 and PC-3 cells with CI calculated well under 1 (**Figure 45**). Additionally using either agent in a 3:1 ratio also showed strong synergism in the tested cell lines. A 3:1 docetaxel to LQZ-7F-1 ratio displayed the lowest CI value in both PC-3 and C4-2 cells lines. The combination experiment results are detailed in the isobologram analysis (**Figure 45D and E**). This data provides support that a combination therapy between LQZ-7F-1 and docetaxel may be beneficial and synergize in prostate cancer *in vivo* models.

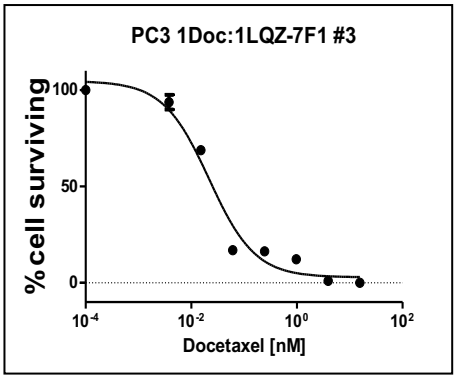
A

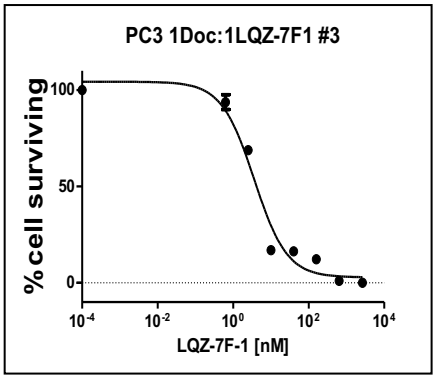

PC3 1Doc:1LQZ-7F1 #1
% cell surviving
100
50
0
10^{-4}
10^{-2}
10^{0}
10^{2}
Docetaxel [nM]

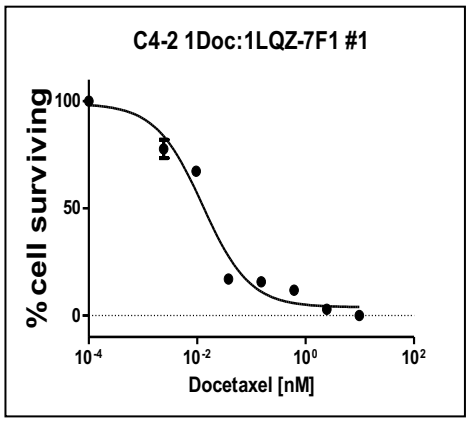

PC3 1Doc:1LQZ-7F1 #1
% cell surviving
100
50
0
10^{-4}
10^{-2}
10^{0}
10^{2}
10^{4}
LQZ-7F-1 [nM]

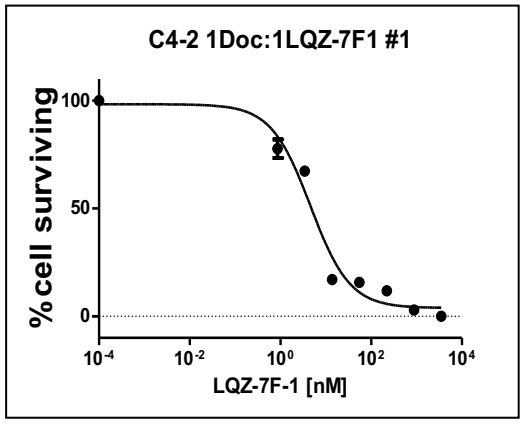

PC3 1Doc:1LQZ-7F1 #2
% cell surviving
100
50
0
10^{-4}
10^{-2}
10^{0}
10^{2}
Docetaxel [nM]

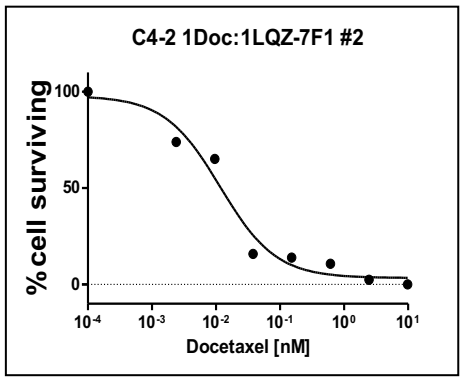

PC3 1Doc:1LQZ-7F1 #2
% cell surviving
100
50
0
10^{-4}
10^{-2}
10^{0}
10^{2}
10^{4}
LQZ-7F-1 [nM]

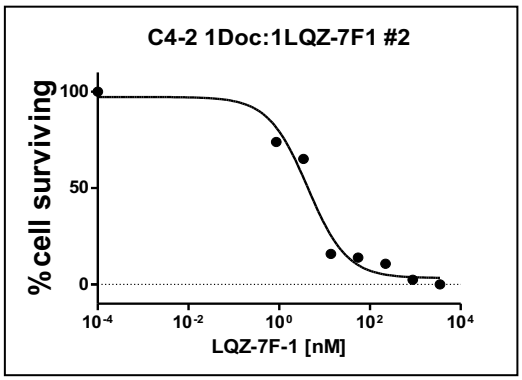

PC3 1Doc:1LQZ-7F1 #3
% cell surviving
100
50
0
10^{-4}
10^{-2}
10^{0}
10^{2}
Docetaxel [nM]

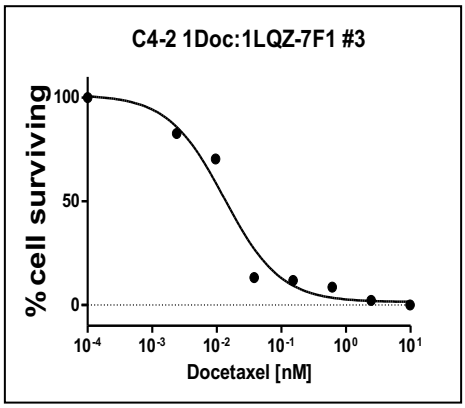

PC3 1Doc:1LQZ-7F1 #3
% cell surviving
100
50
0
10^{-4}
10^{-2}
10^{0}
10^{2}
10^{4}
LQZ-7F-1 [nM]

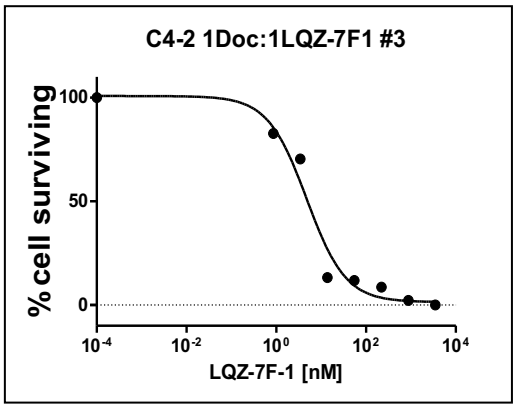

C4-2 1Doc:1LQZ-7F1 #1
% cell surviving
100
50
0
10-4
10-2
100
102
Docetaxel [nM]


C4-2 1Doc:1LQZ-7F1 #1
% cell surviving
100
50
0
10-4
10-2
100
102
104
LQZ-7F-1 [nM]

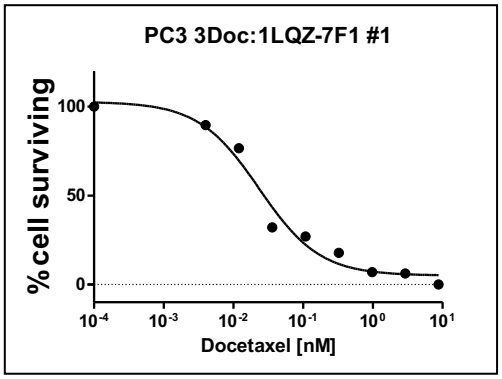

C4-2 1Doc:1LQZ-7F1 #2
% cell surviving
100
50
0
10-4
10-3
10-2
10-1
100
101
Docetaxel [nM]

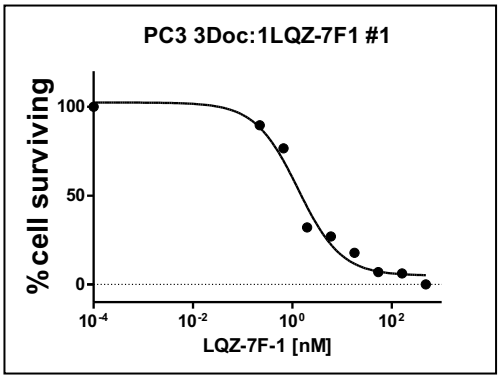

C4-2 1Doc:1LQZ-7F1 #2
% cell surviving
100
50
0
10-4
10-2
100
102
104
LQZ-7F-1 [nM]

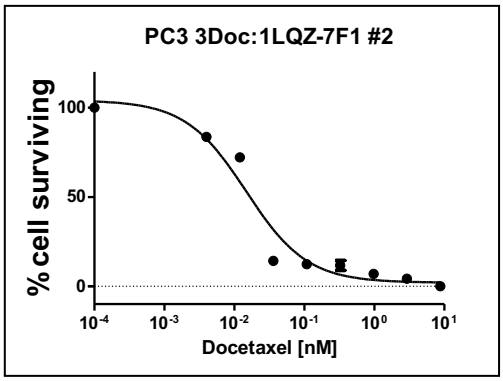

C4-2 1Doc:1LQZ-7F1 #3
% cell surviving
100
50
0
10-4
10-3
10-2
10-1
100
101
Docetaxel [nM]

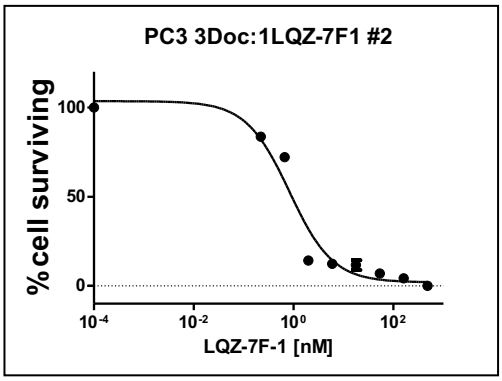

C4-2 1Doc:1LQZ-7F1 #3
% cell surviving
100
50
0
10-4
10-2
100
102
104
LQZ-7F-1 [nM]

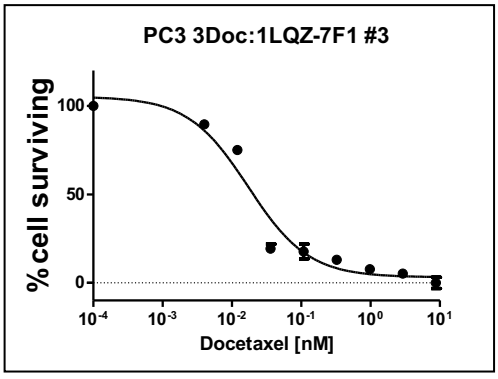
B

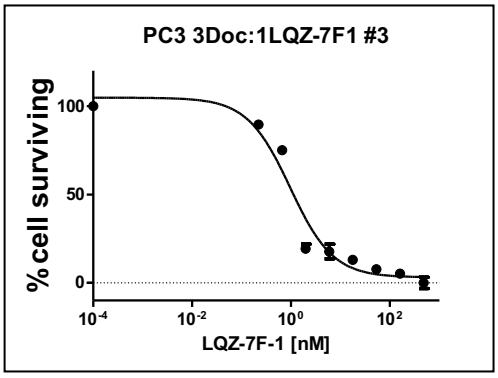

PC3 3Doc:1LQZ-7F1 #1
% cell surviving
100
50
0
10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1}
Docetaxel [nM]

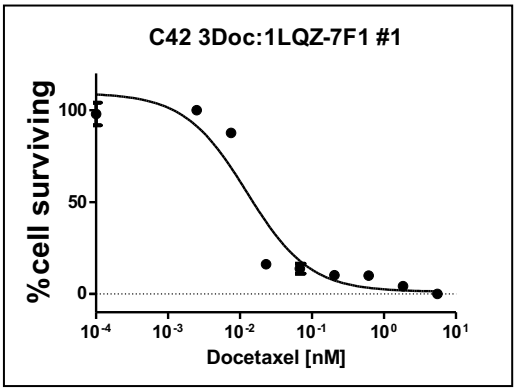

PC3 3Doc:1LQZ-7F1 #1
% cell surviving
100
50
0
10^{-4} 10^{-2} 10^{0} 10^{2}
LQZ-7F-1 [nM]

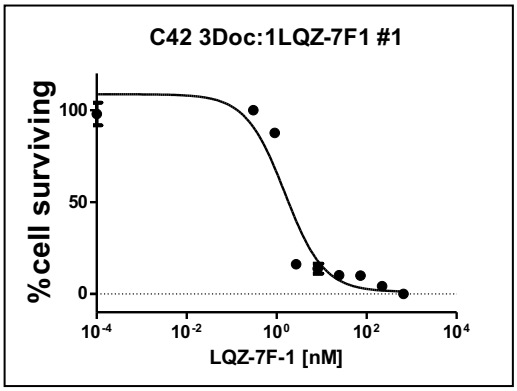

PC3 3Doc:1LQZ-7F1 #2
% cell surviving
100
50
0
10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1}
Docetaxel [nM]

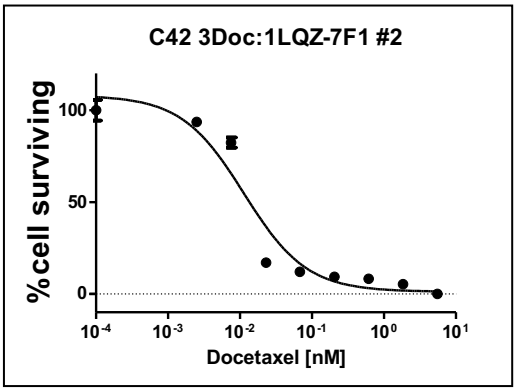

PC3 3Doc:1LQZ-7F1 #2
% cell surviving
100
50
0
10^{-4} 10^{-2} 10^{0} 10^{2}
LQZ-7F-1 [nM]

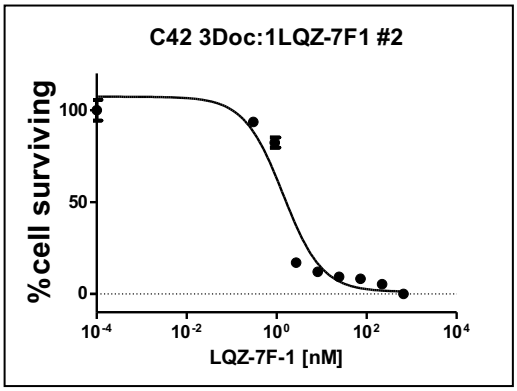

PC3 3Doc:1LQZ-7F1 #3
% cell surviving
100
50
0
10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1}
Docetaxel [nM]

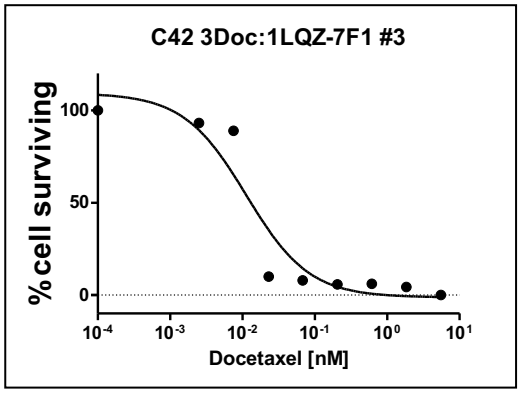

PC3 3Doc:1LQZ-7F1 #3
% cell surviving
100
50
0
10^{-4} 10^{-2} 10^{0} 10^{2}
LQZ-7F-1 [nM]

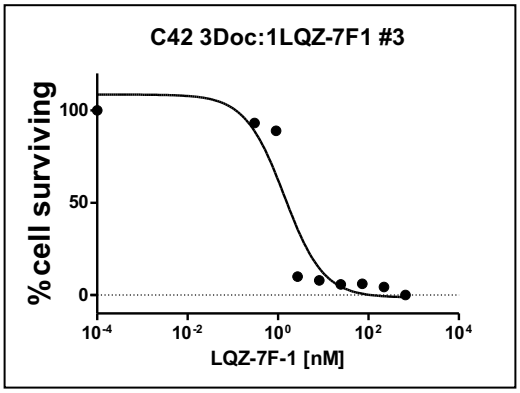

C42 3Doc:1LQZ-7F1 #1
%cell surviving
100
50
0
10-4
10-3
10-2
10-1
100
101
Docetaxel [nM]


C42 3Doc:1LQZ-7F1 #1
%cell surviving
100
50
0
10-4
10-2
100
102
104
LQZ-7F-1 [nM]

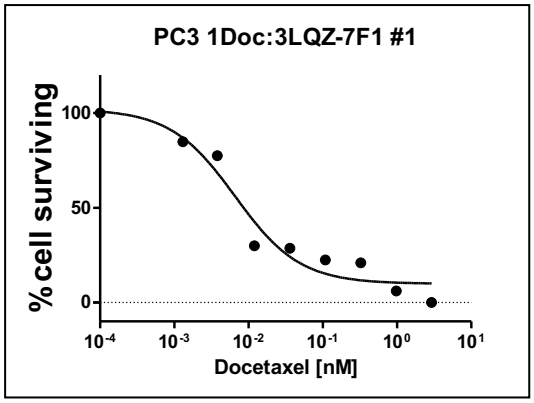

C42 3Doc:1LQZ-7F1 #2
%cell surviving
100
50
0
10-4
10-3
10-2
10-1
100
101
Docetaxel [nM]

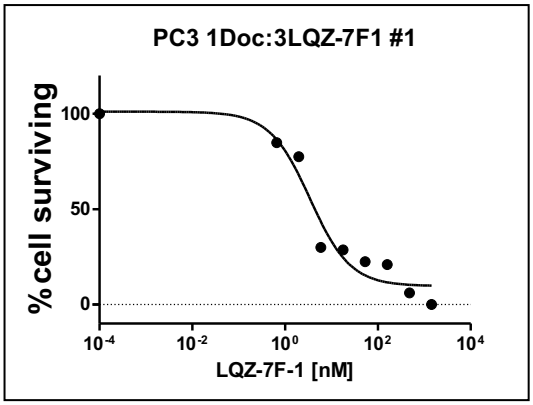

C42 3Doc:1LQZ-7F1 #2
%cell surviving
100
50
0
10-4
10-2
100
102
104
LQZ-7F-1 [nM]

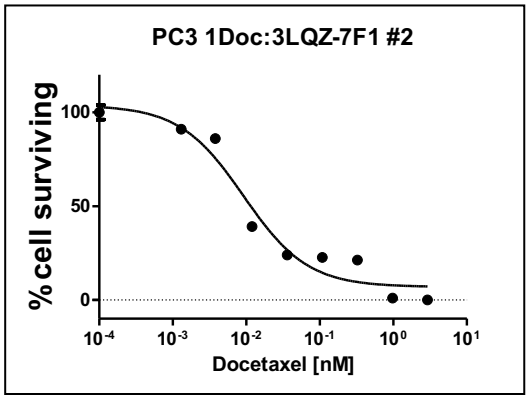

C42 3Doc:1LQZ-7F1 #3
%cell surviving
100
50
0
10-4
10-3
10-2
10-1
100
101
Docetaxel [nM]

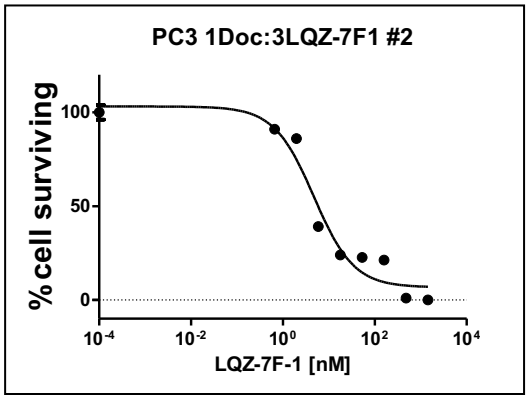

C42 3Doc:1LQZ-7F1 #3
%cell surviving
100
50
0
10-4
10-2
100
102
104
LQZ-7F-1 [nM]

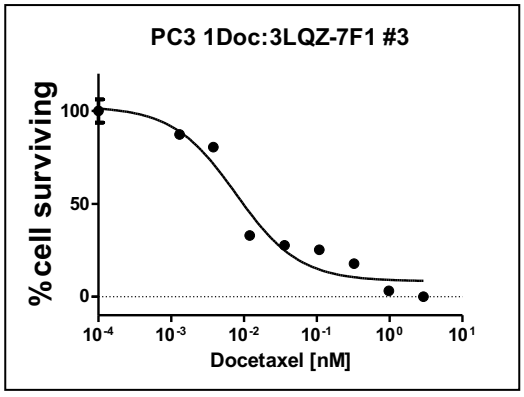
C

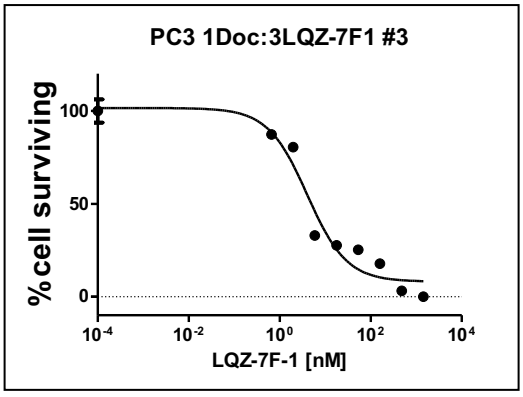

PC3 1Doc:3LQZ-7F1 #1
% cell surviving
100
50
0
10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1}
Docetaxel [nM]

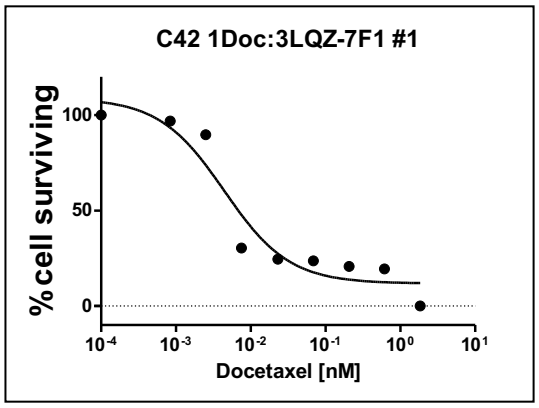

PC3 1Doc:3LQZ-7F1 #1
% cell surviving
100
50
0
10^{-4} 10^{-2} 10^{0} 10^{2} 10^{4}
LQZ-7F-1 [nM]

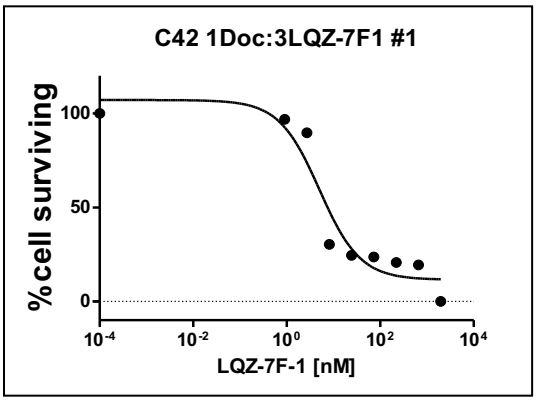

PC3 1Doc:3LQZ-7F1 #2
% cell surviving
100
50
0
10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1}
Docetaxel [nM]

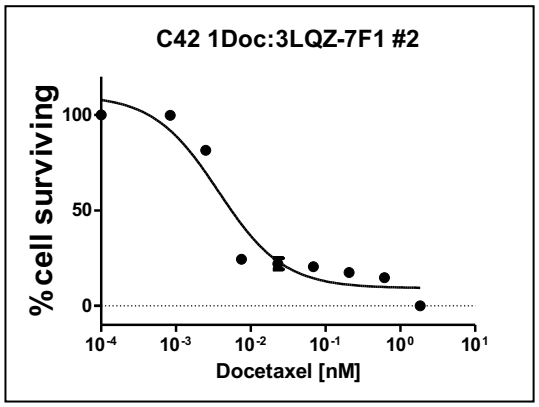

PC3 1Doc:3LQZ-7F1 #2
% cell surviving
100
50
0
10^{-4} 10^{-2} 10^{0} 10^{2} 10^{4}
LQZ-7F-1 [nM]

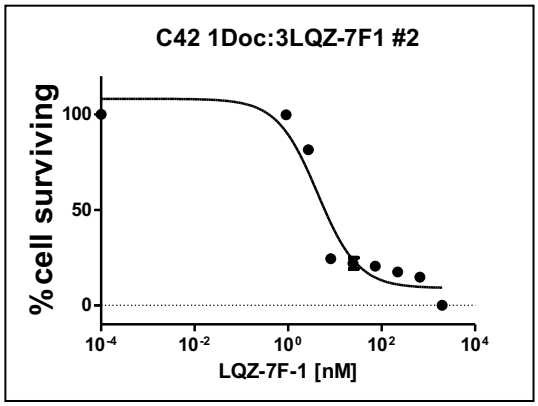

PC3 1Doc:3LQZ-7F1 #3
% cell surviving
100
50
0
10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1}
Docetaxel [nM]

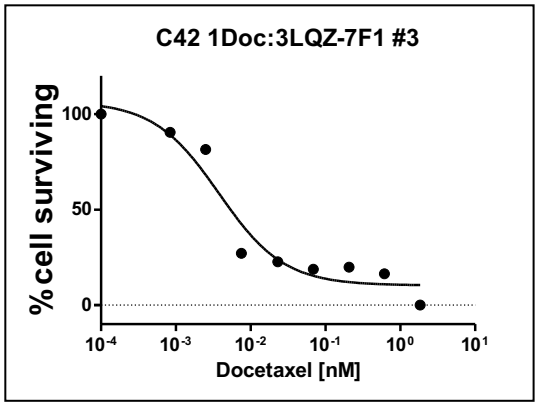

PC3 1Doc:3LQZ-7F1 #3
% cell surviving
100
50
0
10^{-4} 10^{-2} 10^{0} 10^{2} 10^{4}
LQZ-7F-1 [nM]

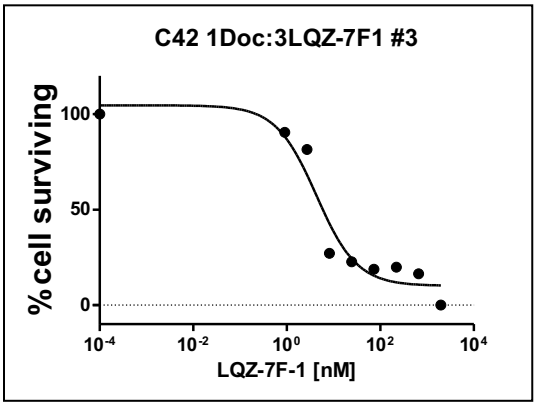

C42 1Doc:3LQZ-7F1 #1
% cell surviving
100
50
0
10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1}
Docetaxel [nM]


C42 1Doc:3LQZ-7F1 #1
% cell surviving
100
50
0
10^{-4} 10^{-2} 10^{0} 10^{2} 10^{4}
LQZ-7F-1 [nM]

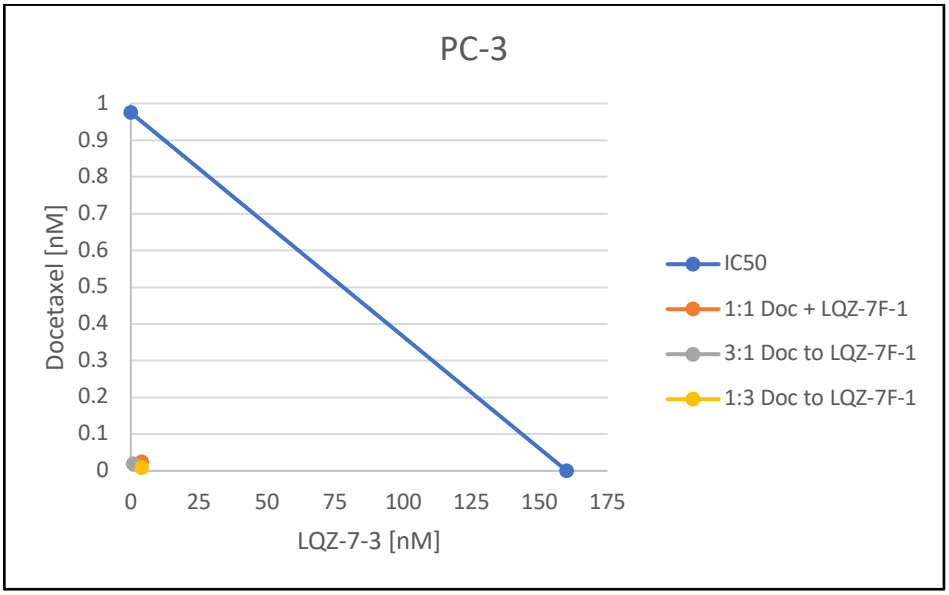

C42 1Doc:3LQZ-7F1 #2
% cell surviving
100
50
0
10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1}
Docetaxel [nM]


C42 1Doc:3LQZ-7F1 #2
% cell surviving
100
50
0
10^{-4} 10^{-2} 10^{0} 10^{2} 10^{4}
LQZ-7F-1 [nM]

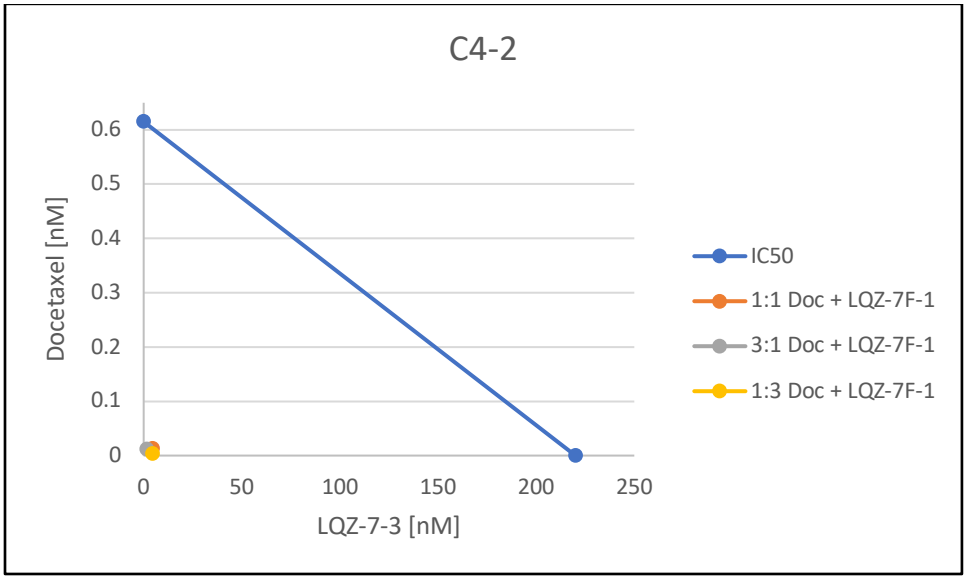

C42 1Doc:3LQZ-7F1 #3
% cell surviving
100
50
0
10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1}
Docetaxel [nM]


C42 1Doc:3LQZ-7F1 #3
% cell surviving
100
50
0
10^{-4} 10^{-2} 10^{0} 10^{2} 10^{4}
LQZ-7F-1 [nM]

D


PC-3
Docetaxel [nM]
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
25
50
75
100
125
150
175
LQZ-7-3 [nM]
IC50
1:1 Doc + LQZ-7F-1
3:1 Doc to LQZ-7F-1
1:3 Doc to LQZ-7F-1

E


C4-2
Docetaxel [nM]
0.6
0.5
0.4
0.3
0.2
0.1
0
0
50
100
150
200
250
LQZ-7-3 [nM]
IC50
1:1 Doc + LQZ-7F-1
3:1 Doc + LQZ-7F-1
1:3 Doc + LQZ-7F-1

F

Ratio (IC_{50}:IC_{50})	C4-2	PC-3
1 to 1	0.041	0.051
3 Doc:1 LQZ-7F-1	0.026	0.025
1 Doc:3 LQZ-7F-1	0.0273	0.034

Figure 45. **LQZ-7F-1 synergizes with docetaxel in vitro.** Methylene response curves in combination treatments of docetaxel and LQZ-7F-1 in (A) 1:1 ratio, (B) 3 Doc:1 LQZ-7F-1 ratio, (C) 1 Doc:3 LQZ-7F-1 ratio. Each data point was performed in triplicate. (D) PC-3 and (E) C4-2 cells Isobologram analysis demonstrating strong synergism between docetaxel and LQZ-7F-1 in. Each point represents the average of three independent experiments. (F) Summarized CI value for both cell lines with different ratios tested.

5.2.10 LQZ-7F Structure Activity Relationship Analysis

The design and synthesis of the LQZ-7F structural analogues was performed to allow for information to be garnered from the effect of different functional group additions to one specific position of the backbone of LQZ-7F. Data from the single concentration analysis in three different cell lines was utilized to specifically compare the effect of additions of different moieties to the cyclopentane group in the LQZ-7F backbone. As shown in **Figure 46**, there was clear evidence that the addition of a carbonyl functional group to the cyclopentane ring lead to a significantly more potent compound in terms of cell cytotoxicity and ability to inhibit dimerization in the two hybrid assay than other analogues. No other moieties increased the performance of any of the other analogues in comparison to LQZ-7F. In fact almost all of the analogues performed significantly worse than LQZ-7F in this series of experiments. Overall, the carbonyl group addition to form a cyclopentanone in the LQZ-7F-1 backbone established a compound with significantly lower IC_{50} value and increased effect on target protein survivin.

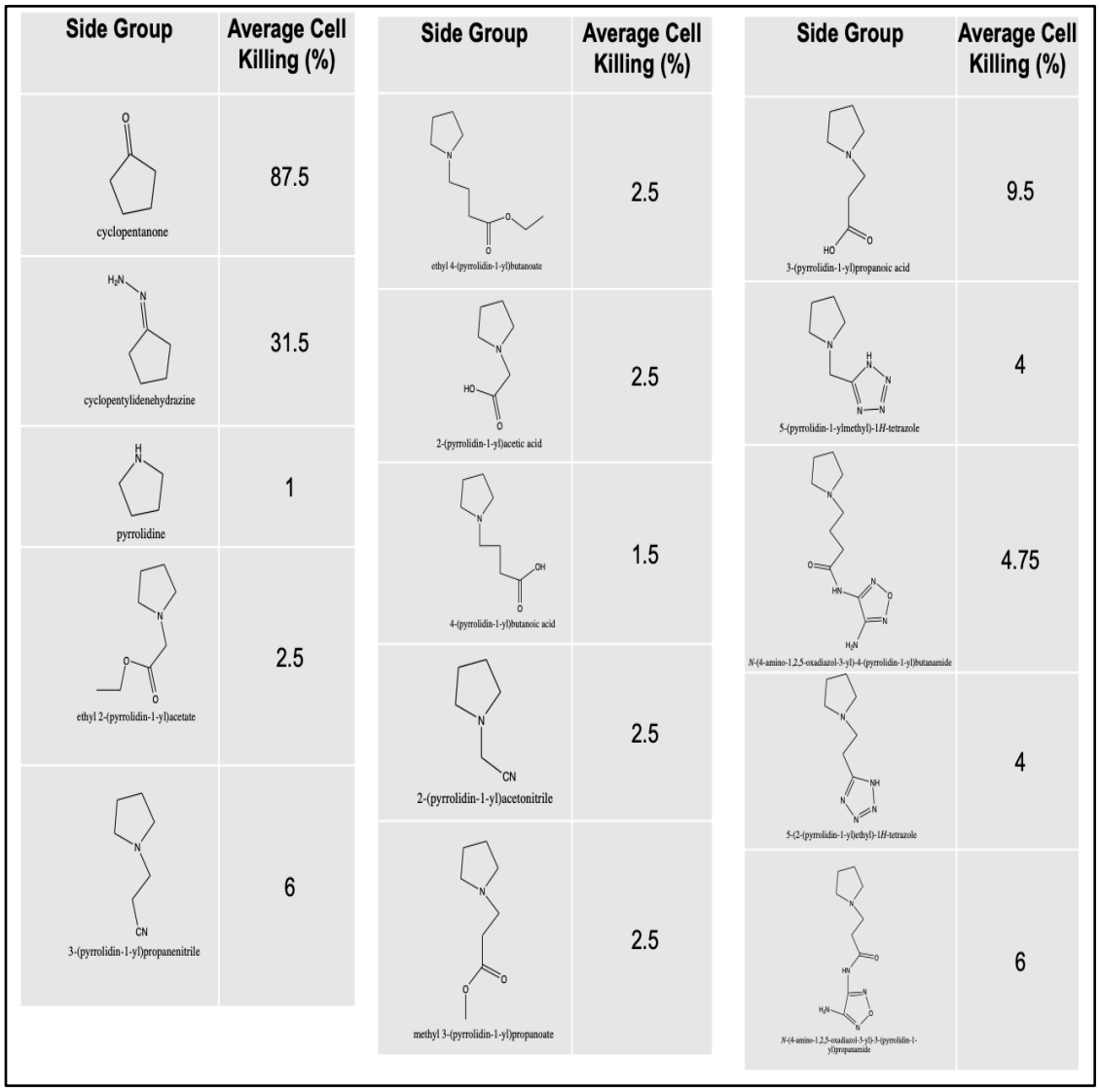


Figure 46. **Functional group analysis of analogue changes at the cyclopentane group position of LQZ-7F.** LQZ-7F-1 has a carbonyl added to the cyclopentane had enhanced cytotoxicity in cell lines as well as improved performance in dimerization assay compared to other functional group changes at the same position.

5.2.11 Generation of LQZ-7F-1 Structural Analogues

The next iterative generation of survivin inhibitors were structural analogues of LQZ-7F-1 and were synthesized with changes that can be grouped into three broad categories. The first group of analogues have additional functional group changes at the cyclopentane group. The second group involves changes to the oxadiazine ring in the backbone. The final group involves the addition of different functional groups to the benzene ring in the LQZ-7F-1 backbone. The idea behind this round of chemical synthesis of structural analogues was to garner further information on critical components of LQZ-7F-1 compound that are critical for its function. The thirty structural analogues of LQZ-7F-1 are shown in **Figure 47**.

5.2.12 LQZ-7F-1 Analogues Single Concentration Analysis

In order to determine if any of the LQZ-7F-1 structural analogues had a greater ability to promote cancer cell killing than LQZ-7F-1, a single concentration analysis was first performed in PC-3 and C4-2 cells. For this series of experiments, cells were treated with 150 nM of each different compound for 72 hours. This concentration was specifically chosen as it represents roughly the average IC_{50} value of LQZ-7F-1 in prostate cancer cells. The results from the single concentration methylene blue assays are shown in **Figure 48**. As excepted, treatment of LQZ-7F-1 at 150 μM inhibited both PC-3 and C4-2 roughly 50-60%. While many analogues displayed activity, none generated in this round of synthesis had a greater cell killing effect than LQZ-7F-1. The data in this

section further positioned LQZ-7F-1 as the compound to use in future studies while also providing structure activity relationship insights in its backbone.

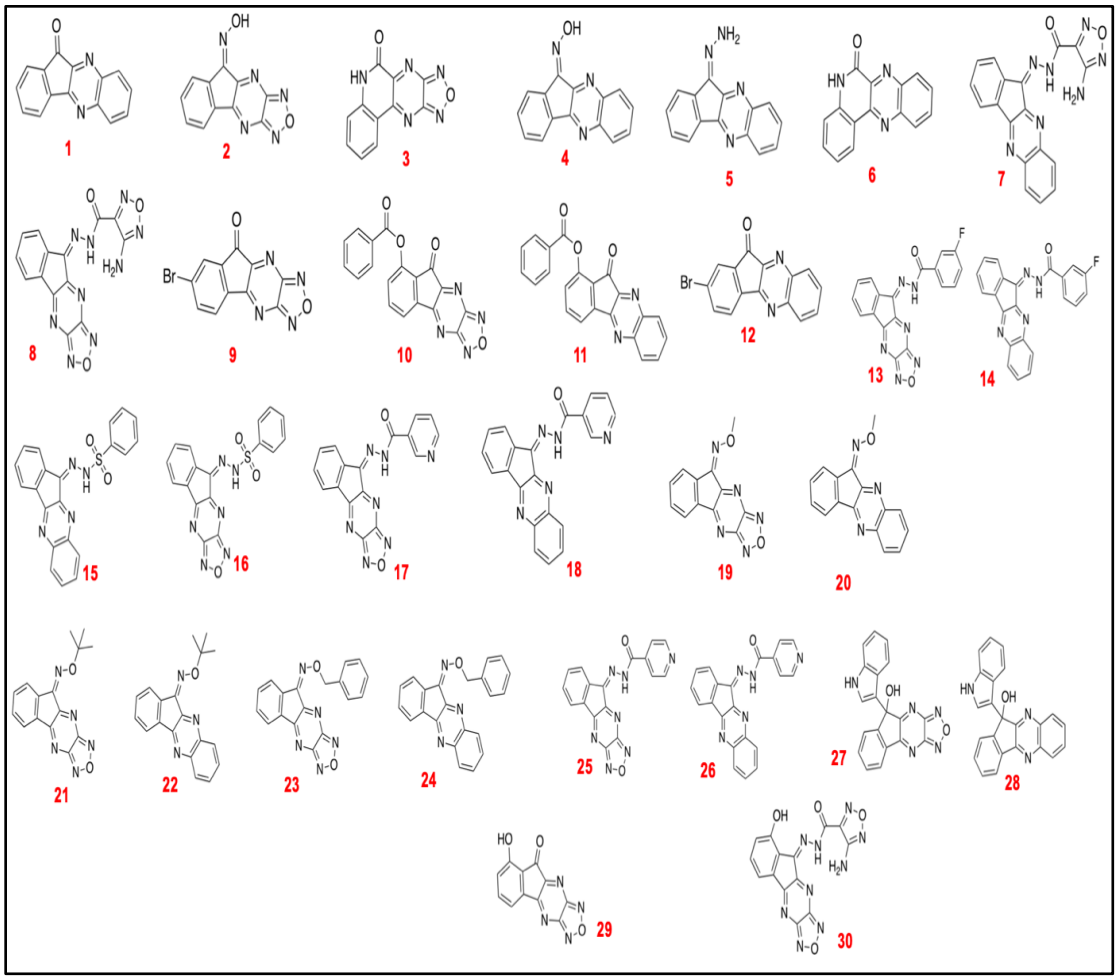
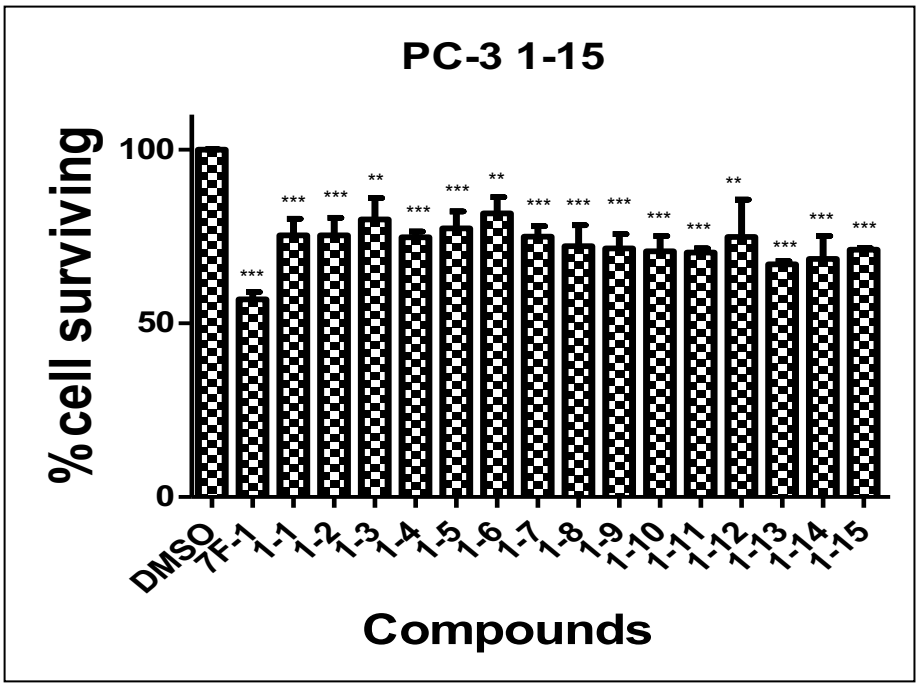
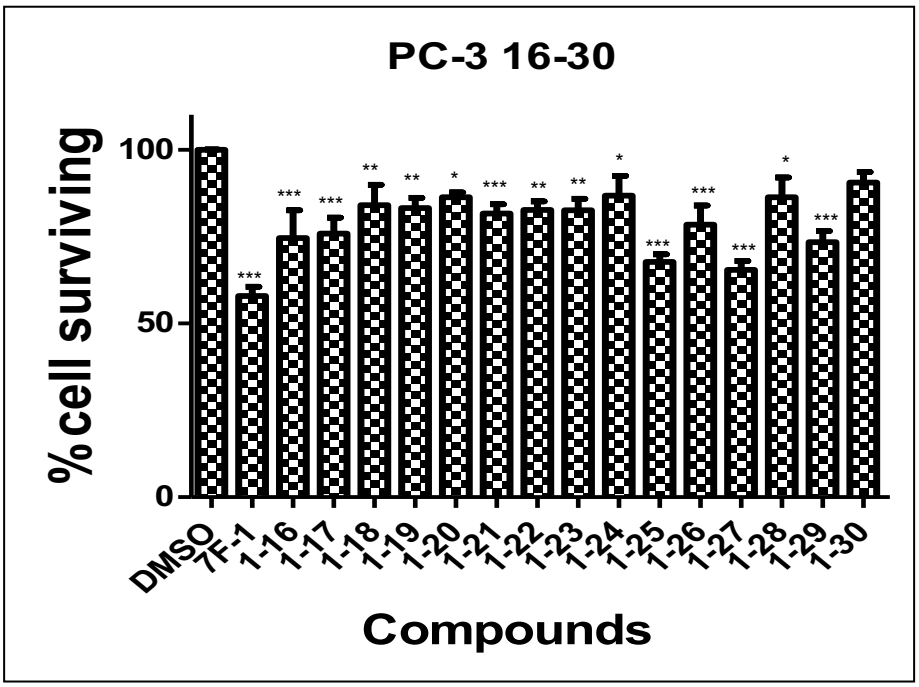


Figure 47. **Chemical structures of LQZ-7F-1 analogues with substitutions at cyclopentane group, oxadiazine ring, or benzene ring of the parental compound's backbone.**

A


PC-3 1-15
% cell surviving
100
50
0
DMSO
7F-1
1-1
1-2
1-3
1-4
1-5
1-6
1-7
1-8
1-9
1-10
1-11
1-12
1-13
1-14
1-15
Compounds


PC-3 16-30
% cell surviving
100
50
0
DMSO
7F-1
1-16
1-17
1-18
1-19
1-20
1-21
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1-23
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1-25
1-26
1-27
1-28
1-29
1-30
Compounds

B

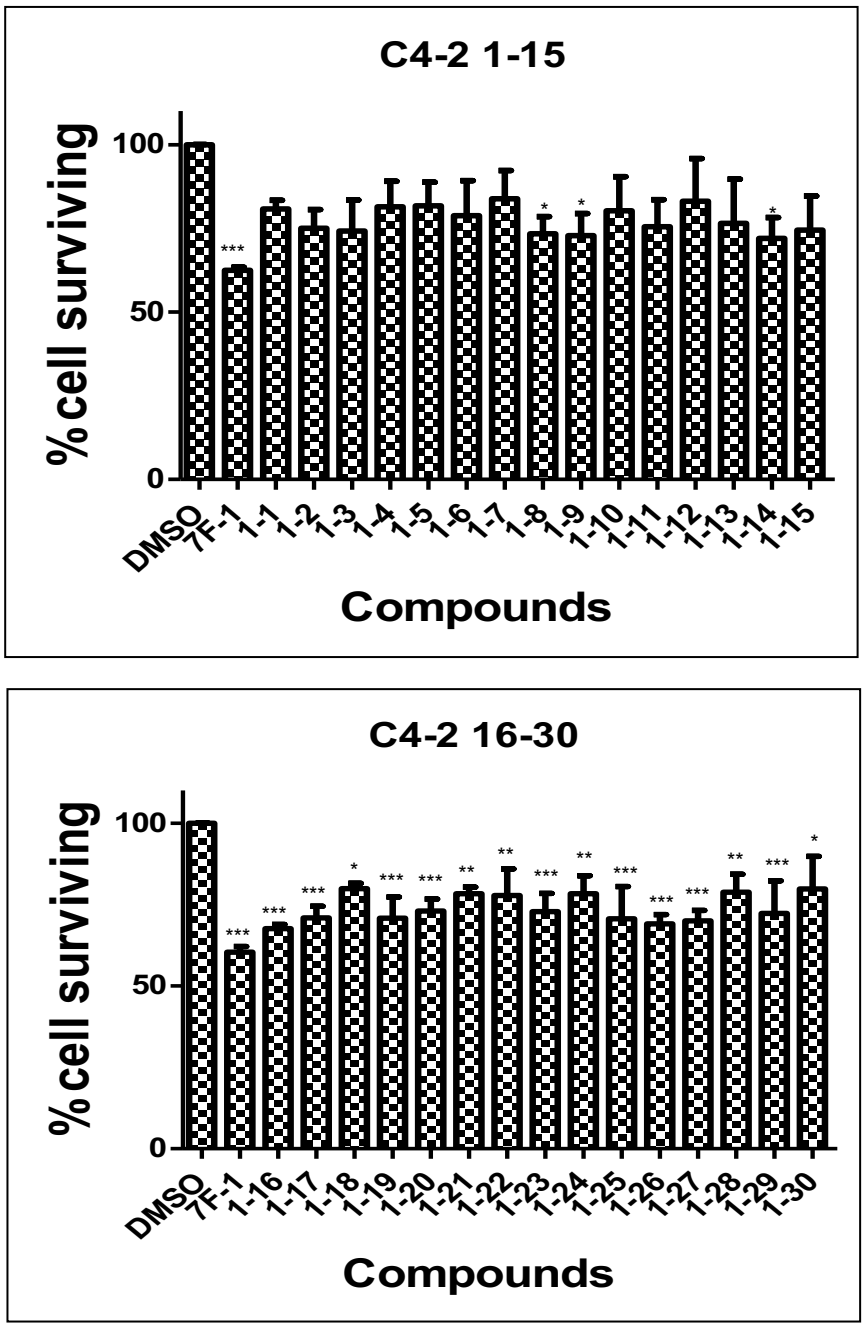


Figure 48. **Single concentration analysis of LQZ-7F-1 and structural analogues.** (A) PC-3 and (B) C4-2 cells were treated with 150 nM of each compound for 72 hours. The results indicate the percentage cells surviving after treatment. No compound had greater amount of cell killing than parental LQZ-7F-1. Each concentration was tested in triplicate *** = p-value <0.001. n = 3 independent experiments. Error bar equals standard deviation.

5.2.13 LQZ-7F-1 Structure Activity Relationship Analysis

Although the synthesis of 30 structural analogues did not yield an improved compound in terms of performance in cell based assays, the strategic alterations at specific positions of LQZ-7F-1 helped further elucidate critical aspects of the compound. As detailed in **Figure 49**, there were three primary takeaways from the structure activity relationship analysis that was performed. First, the addition of the carbonyl group to the cyclopentane group (LQZ-7F-1) generated the most potent inhibitor in cytotoxicity and survivin inhibition assays. Secondly, the compounds that contained the oxadiazine ring in the ring backbone were more potent inhibitors of cell survival than those with a benzene ring at the same position. Finally, no functional group addition to the benzene ring of the backbone increased the potency of any of the inhibitors tested.

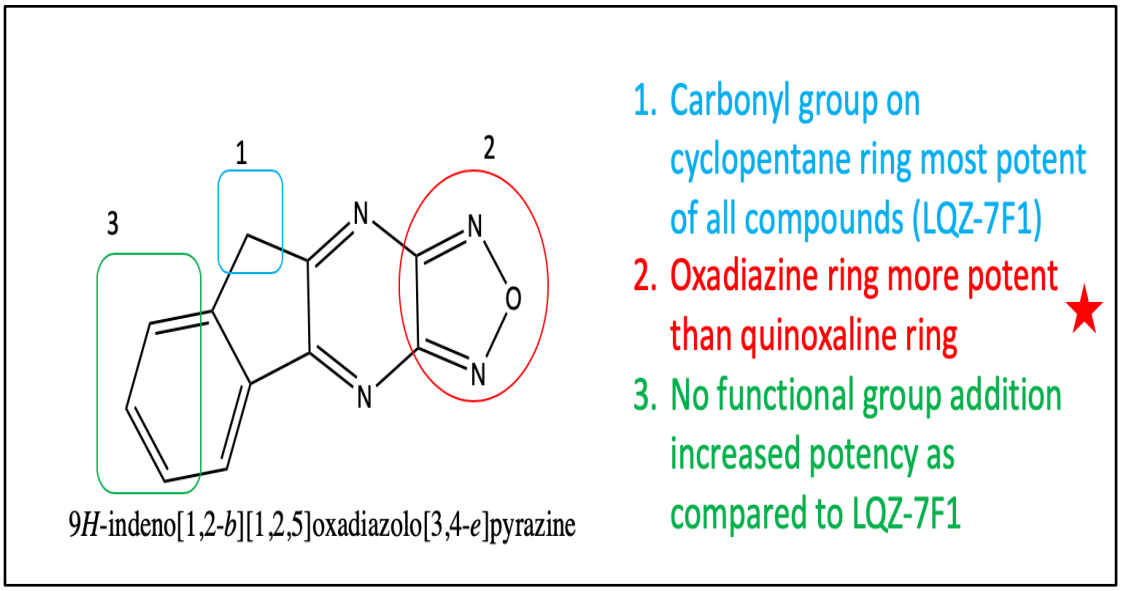


Figure 49. **Major takeaways from structural analysis of LQZ-7F and LQZ-7F-1 rounds of analogue generation.** (1) The carbonyl group addition to the cyclopentane group (LQZ-7F-1 compound) formed the most potent compound. (2) Compounds with the oxadiazine ring were more potent than compounds with quinoxaline ring at the same position. (3) No functional group addition to the benzene ring of the backbone increases potency in cell based assays.

5.3 Concluding Remarks

The data in this section serve as a first generation optimization of the locked LQZ-7F survivin dimerization inhibitor backbone. In the experiments above, a structural analogue of LQZ-7F was found that has enhanced cytotoxicity in prostate cancer cells and inhibits survivin dimerization in a mammalian two hybrid assay greater than the parental compound. The compound, LQZ-7F-1, caused significant survivin degradation earlier than 8 hours in both PC-3 and C4-2. The loss of survivin caused by LQZ-7F-1 treatment is blocked by pretreatment with proteasome inhibitors, suggesting LQZ-7F-1 induced survivin loss is via proteasome degradation. LQZ-7F-1 increases cellular apoptosis of prostate cancer cells compared to control as measured by annexin V staining and increases in cleaved caspase 3 protein levels. Importantly, LQZ-7F-1 synergizes with docetaxel, warranting the exploration of a combination therapy in future xenograft efficacy studies. Finally, the carbonyl group of LQZ-7F-1, which was added to the cyclopentane in LQZ-7F, confers improved cellular responses in multiple assays as compared to other functional group substitutions at the same position.

CHAPTER 6. DISCUSSION

6.1 Summary of Findings and Discussion

Based on literature findings and data presented in **Chapter 3**, survivin appears to be a major contributor to docetaxel resistance. As summarized in **Figure 50**, based on data from **Chapters 4-5,** novel survivin inhibitors that block or disrupt survivin dimerization may lead to survivin protein degradation via the proteasome. The loss in survivin results in a decrease in survivin's cellular functions and increased cell death by apoptosis. Direct survivin dimerization inhibitors may sensitize resistant cancer cells to docetaxel. Detailed summaries and discussion follow below.

6.1.1 Survivin Contributes to Docetaxel Resistance in Prostate Cancer Cells

The data from **Chapter 3** served to implicate survivin as an important molecular player in docetaxel resistance in prostate cancer. The survivin expression level in the five different prostate cancer cell lines tested had a strong positive correlation with docetaxel cytotoxicity. The same was not true for other solid tumor chemotherapeutics cisplatin and doxorubicin. Additionally, castration resistant and more aggressive cell lines had significantly higher survivin expression levels than androgen dependent LNCAP cells. In the in vitro model of stepwise docetaxel selection, docetaxel resistant cells showed significantly increased survivin expression to go along with their resistance to docetaxel. Comparatively, parental cells were sensitive to docetaxel and had lower survivin expression levels. In prostate cancer cells the stable overexpression of survivin

increased resistance to docetaxel and knockdown of survivin in docetaxel resistant cells partially restored their response to docetaxel treatment. The knockdown did not entirely return these cells to the sensitivity seen in the parental cell lines; however this can be contributed to the selection process invoking other factors of resistance besides survivin. It's possible this process could lead to increases in the expression of drug efflux pumps like p-glycoprotein or other anti-apoptotic proteins such as Bcl-2 or Bcl-xl. A more suitable model for exploring the mechanism behind survivin specifically in the context of docetaxel resistance may be the use of patient-derived xenografts that over time are serially exposed to docetaxel and develop resistance. Although the detailed molecular mechanism of how survivin may mediate chemotherapeutic resistance continues to elude the field as a whole, the data presented in **Chapter 3** serves to implicate survivin in the development of docetaxel resistance. More importantly, this data implicates survivin as a molecular player in docetaxel resistance. Further experiments are warranted to determine if inhibition of survivin may help restore cancer cells sensitivity to docetaxel treatment.

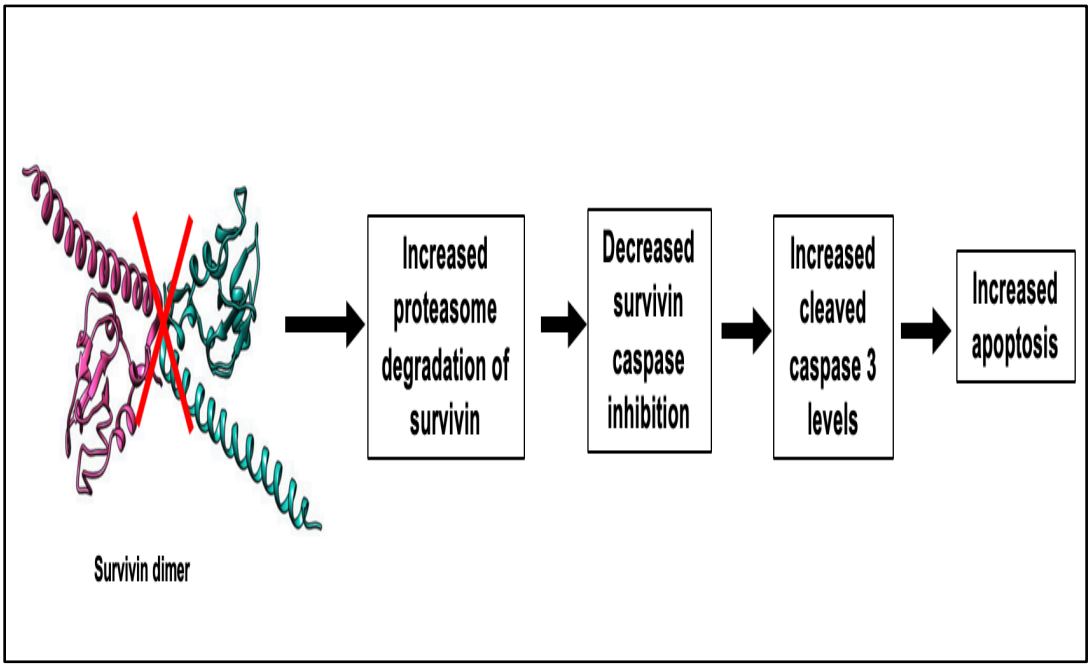


Figure 50. **Summary of survivin inhibitors actions on prostate cancer cells.** Survivin inhibitors targeting the dimerization interface of survivin, block or disrupt dimerization leading to increased degradation of survivin via the proteasome. The decrease in survivin leads to decreased survivin inhibition of caspase activation. This results in increased cleaved caspase levels and increased apoptosis leading to death of cancer cells.

6.1.2 LQZ-7-3 Inhibits Survivin Greater than its Predecessor and Reduces Prostate Cancer Xenograft Growth *In Vivo*

The data in **Chapter 4** served as a first generation optimization of the LQZ-7 survivin dimerization inhibitor backbone that was discovered as the primary hit in our original *in silico* screening for compounds that could interact with the key core dimerization residues of survivin. A structural analogue of LQZ-7 was found that has improved cytotoxicity in prostate cancer cells and inhibited survivin dimerization in a mammalian two hybrid assay greater than the parental compound. The compound, LQZ-7-3, also had a strong positive correlation between survivin expression and cytotoxicity in five different prostate cancer cell lines. In an IAP family degradation assay, LQZ-7-3 showed specificity toward promoting survivin degradation while not affecting other members of the IAP family's protein level.

Survivin degradation after LQZ-7-3 treatment occurred earlier than 24 hours in both PC-3 and C4-2. Treatment with LQZ-7-3 also significantly reduced the half-life of survivin to well under one hour in both cell lines. Survivin degradation caused by treatment with LQZ-7-3 appears to be via the proteasome as two different proteasome inhibitors blocked the loss of survivin and restored the levels to that seen in the control treated cells. The loss of survivin by LQZ-7-3 treatment appears to lead to spontaneous apoptosis of cancer cells as measured by annexin v staining and increased caspase 3 levels. In a mouse xenograft efficacy study, LQZ-7-3 reduced tumor volume after administration of ten oral treatments as compared to vehicle control. It's important to note that

pharmacokinetic studies need to be performed on this compound to ensure optimal compound concentrations are being reached. Since tumors were not entirely eliminated in this study, LQ-7-3 may better positioned in this formulation to be a part of a combination treatment. In both PC-3 and C4-2 cells, the use of docetaxel and LQZ-7-3 in a one to one ratio at the IC_{50} of each drug lead to strong synergism. The data provided by the CI studies warrants a future efficacy study using docetaxel and LQZ-7-3 in combination to try to see a more robust elimination of prostate cancer tumors in the mice.

The meticulous design and synthesis of analogues with specific changes to the LQZ-7 backbone also allowed for a basic structure activity relationship analysis amongst this group of compounds. The addition of fluorinated benzene to the amine group established a significantly more potent compound in terms of cell cytotoxicity and ability to inhibit dimerization in the two hybrid assay than other analogues. Additionally, the substitution of methyl groups to the benzene ring also significantly decreased potency and ability to inhibit survivin dimerization as compared to fluorine atom alone. The addition of more bulky moieties in the other analogues decreased performance overall in the cell based assays. Overall, the fluorinated benzene ring of LQZ-7-3 performed better than any other functional group changes to LQZ-7 backbone. I postulate that this improvement in performance can be attributed to the improved solubility and cell permeability associated with fluorinated small molecules [211]. It is possible the addition of the fluorine moieties to LQZ-7-3 may also increase binding affinity to target protein survivin as seen previously in fluorine medicinal chemistry [212].

Finally, it has been demonstrated fluorinated small molecules often have increased intrinsic potency and better pharmacokinetic properties than non-fluorinated counterparts [213]. All of the possibilities mentioned above may contribute to LQZ-7-3 improved performance in assays and warrant further exploration.

6.1.3 LQZ-7F-1 is a Potent Survivin Inhibitor in the Nanomolar Range

LQZ-7F was a special analogue generated from LQZ-7 that had distinct backbone unlike other compounds identified in previous screenings. This backbone consisted of four locked ring structures that result in a less flexible compound backbone. The data in **Chapter 5** served as a first generation optimization of the locked LQZ-7F survivin dimerization inhibitor backbone. LQZ-7F-1, a structural analogue of LQZ-7F, was found that has increased cytotoxicity in prostate cancer cells and inhibited survivin dimerization in a mammalian two hybrid assay greater than LQZ-7F. The compound, LQZ-7F-1, caused significant survivin degradation earlier than 8 hours in both PC-3 and C4-2. Similarly to LQZ-7-3, pretreatment with proteasome inhibitors rescued and restored survivin loss by LQZ-7F-1 to that of control levels. This further indicated that the class of survivin inhibitors presented here cause survivin degradation via the proteasome. The loss of survivin by LQZ-7F-1 also increased caspase 3 levels and lead to apoptosis of prostate cancer cells. As the data presented in the previous sections has indicated the possibility of survivin inhibitors being more successful in a combination therapy, it was promising to see strong synergism between

docetaxel and LQZ-7F-1 In the structure activity relationship analysis, the carbonyl group of LQZ-7F-1 which was added to the cyclopentane in LQZ-7F showed significant increases in potency obtaining a nanomolar IC_{50}. The similarity between LQZ-7F and LQZ-7F-1 leads us to hypothesize that LQZ-7F-1 may be an active metabolite product of LQZ-7F metabolism. This possibility is exciting and warrants further assessment in future studies. Overall, the strong performance of LQZ-7F-1 cell based assay activity warrants further investigation as a lead compound in efficacy studies.

6.2 Points of Consideration

In this study, survivin was emphasized as an important cellular player in docetaxel resistance, and two primary lead compounds were identified for future efficacy studies as a single agent and combination therapy with docetaxel. There were not major obstacles encountered during the completion of this thesis besides minor technical adjustments required to optimize protocols. However, there may exist some potential limitations associated with the experiments presented here that warrant further discussion and evaluation going forward.

The use of 2D monolayer cell culture provides an easy, comparative method for the growth and analysis of cancer cells in culture. While 2D cultures have advanced the fields knowledge and allowed for greater understanding of cancer cell behaviors and mechanisms of resistance, the growth of cells on a flat surface has its limitations when trying to translate discoveries to *in vivo* systems. Currently, 2D cell cultures are often hyper-sensitive to anti-cancer agents and

also fail to the model for the dynamic interactions between cancer cells and their microenvironment. Consequently, these cell line models may not be the best for transitioning results to the physiology of *in vivo* models and actual patients.

There is a still a lot of work to be done, however 3D cultures in the form of spheroids from cell lines and organoids from patient samples may better represent the dynamic interplay that occurs in a tumor. In these 3D cultures an extra-cellular matrix can surround the cells and allows for the interaction between the cancer cells and their microenvironment. The development of organoids from primary tumors or metastases from patients also allow for the study of cancer stem cells and microenvironment while also maintain tumor heterogeneity [214]. 3D cultures generally have the problem that their complexity limits biological replicates and combination therapies are sometimes not practical, however more recently there has been the development of microwell-mesh 3D cultures that allow for the generation of roughly a hundred microtumors per well for screening anti-cancer compounds [215]. In future studies using the survivin inhibitory compounds, the microwell-mesh 3D organoids technique derived from patients may represent innovative tool for therapeutic screening that better reflects the responses in animals and patients.

Finally, the other potential limitation associated with this study is the use of NSG mice for the *in vivo* xenograft efficacy study. These mice are immunodeficient meaning they lack mature T cells, B cells, and NK cells that make up a functioning immune system. Implantation of tumors in these mice are much easier as host rejection of human tumor cells becomes less likely however

this comes with a tradeoff. Since there is a deficiency in the immune system these mouse models are unable to replicate the interplay between the cancer cells, the tumor microenvironment, and mature host immune cells seen in patients. Therefore, there exists the strong possibility that a compound that shows promise and efficacy in these mice may not necessarily work in patients. More recently, researchers have sought to overcome these challenges particularly in prostate cancer. The development of patient-derived xenograft models (PDX) may provide a critical alternative that will allow for clinically relevant and translation data. The PDX models preserve the tumor-microenvironment architecture and also the tumor heterogeneity [216]. Unfortunately, very few PDX models have been successfully established for prostate cancer.

It is likely more appropriate for us going forward to implore an orthotopic syngeneic prostate cancer mouse model which possesses many advantages over subcutaneous injection of cancer cells or use of immunocompromised mice. First, orthotopic implantation involves injecting the cancer cells in to the organ of origin which allows for more clinically accurate vasculature, tumor microenvironment, and response to therapy [217]. There exists a number of established syngeneic mouse models for prostate cancer that allow for elegant experiments such as surgical castration to study castration resistant prostate cancer and even the use of fluorescent cancer cells to monitor there dissemination throughout the body over the course of the study. Syngeneic mouse models also allow for the use of immunocompetent mice and therefore

help maintain the dynamic interplay between cancer cells and host immune cells. Finally, syngeneic mouse models are often times less expensive than genetically engineered models.

6.3 Future Directions

The experiments above demonstrated exciting results like the development of more potent survivin inhibitors that represent the first to directly target the protein itself, as well as survivin appearing to be implicated as a large contributor to docetaxel resistant phenotype seen in prostate cancers. The results provide the foundation for exciting new studies to further our knowledge of docetaxel resistance and survivin's cellular mechanism.

The future studies will focus on the molecular mechanism of survivin mediating docetaxel resistance. For this set of experiments the two stepwise selected docetaxel resistant cell lines, C4-2-Doc and Du145-Doc, as well as their respective parental cell lines as controls would be the primary model utilized. Previously, it was observed that there is a significant overexpression of survivin in docetaxel resistant cell lines as compared to their respective parental cell line. Thus, the first study would be to determine the possible subcellular localization changes of survivin in resistant cells as compared to parental cells using immunofluorescence (IF) and cellular fractionation followed by western blot analysis.

Next, to determine if survivin up-regulation in the docetaxel resistant cells can overcome docetaxel- induced G2/M arrest, knockdown of survivin in the

resistant cells and overexpression of survivin in parental cells would be performed. Then changes in these cells would be compared by FACs cell cycle analysis to see if altering survivin expression level affects docetaxel induced G2/M arrest. As survivin overexpression may mediate faster mitotic progression, one could also test if C4-2-Doc and Du145-Doc cells progress through the cell cycle faster than their parental cells using BrdU pulse labeling followed by FACs analysis. Lastly, to determine the effect of survivin expression on docetaxel binding to microtubules, FACs and IF would be utilized, making use of a fluorescent paclitaxel derivative Flutax-2 as a probe. It is possible that survivin may block or interfere with docetaxel's binding to microtubules and therefore contribute to its ineffectiveness and cells resistance to treatment. The above series of experiments would provide a solid beginning for trying to specifically elucidate the cellular mechanism of survivin in docetaxel resistant prostate cancers.

Lastly, considerable time should be spent to develop improved models for evaluating the therapeutic potential of the survivin inhibitors *in vivo*. For this the use of syngeneic prostate cancer mouse model should be implored. Both LQZ-7-3 and LQZ-7F-1 should undergo formulation optimization for administration in the mice. In many of the previous studies the focus of analogue iterative generations involved improving potency in cell based assays as well as interaction with the survivin dimer. As I continue to develop these inhibitors, it is important to match the improvement in potency with an improved pharmacokinetic profile as well. In our future i*n vivo* work pre-clinical pharmacokinetic assays will be critical to build

the portfolio for these compounds. An improvement in potency or binding affinity to survivin becomes meaningless if these compounds are metabolized to an inactive form or excreted from the body too quickly. Careful study and consideration must be a priority going forward to demonstrate a viable pharmacokinetic profile for each compound. Going forward each compound should be further evaluated in efficacies studies using syngeneic models as a single agent and in particular a combination therapy with docetaxel. These studies would help the pursuit to translate these compounds into a viable anti-cancer treatment option down the road for patients.

6.4 Meaning of Work

The development of resistance to docetaxel and inevitability of metastasis in castration resistant prostate cancer patients represents a significant hurdle to a successful clinical outcome as the disease remains incurable. As stated above, survivin expression has been associated with biologically and clinically aggressive disease and poor patient outcome. The understanding that survivin is likely a large contributor to the resistant phenotype provides strong rationale that targeting survivin may improve patient outcomes and sensitize resistant patients to docetaxel. The long-term goals of this work are and continue to be to understand how survivin contributes to chemotherapy resistance and to use the direct survivin small molecule inhibitors to sensitize tumors to docetaxel. The knowledge gained from the use of direct survivin inhibitors in our first translational studies has helped to identify potential lead candidates for clinical

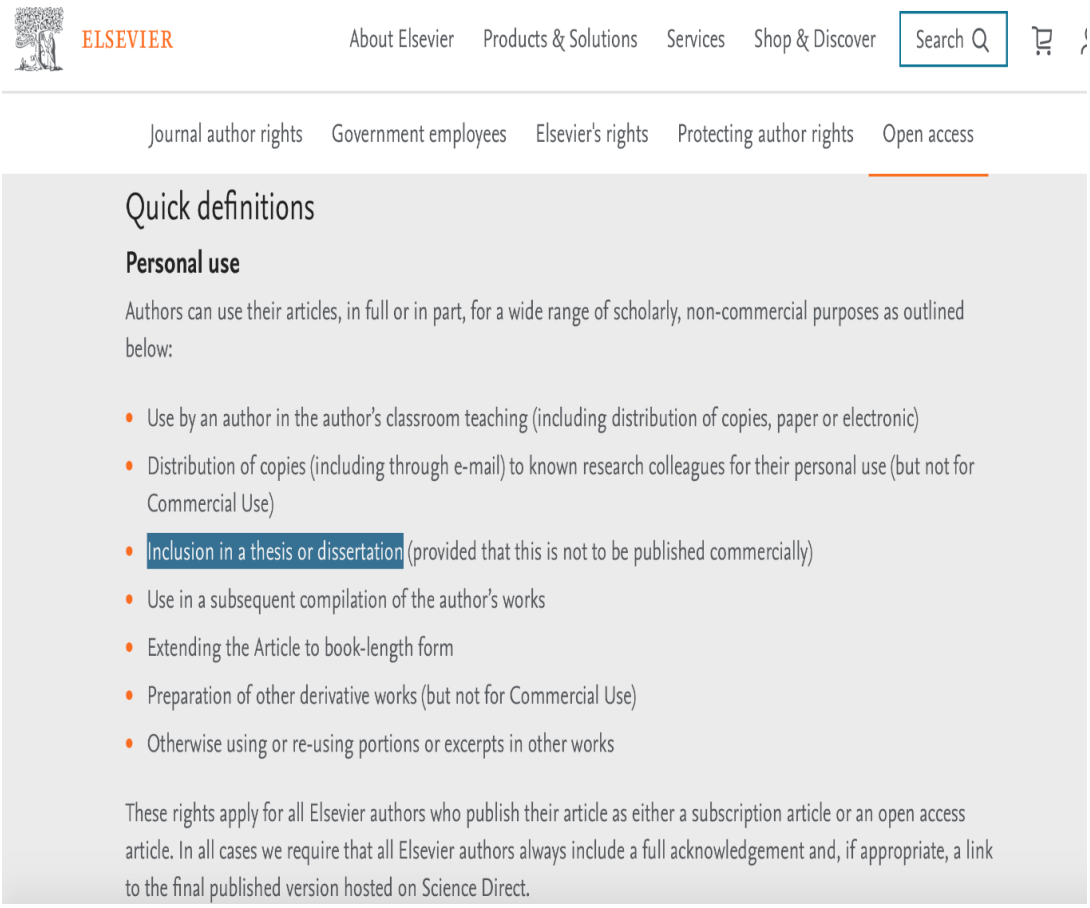
development of inhibitors capable of sensitizing resistant prostate cancer cells to docetaxel. Previous clinical trials with agents designed to manipulate survivin expression have focused on targeting upstream transcription factors or utilizing antisense oligonucleotides and have experienced modest success and often times dose limiting side effects. By directly targeting survivin protein itself, it may be possible to overcome the problems that these other approaches have faced in clinical trials and prove to be a more appropriate option for treatment of CRPC. Finally, almost every solid tumor has been shown to overexpress survivin, therefore the work in this study to develop these survivin inhibitors may not be limited to prostate cancer setting and these inhibitors may have relevance in therapies for other cancer types. Hopefully, the knowledge from this study can eventually help accomplish the ultimate goal of translating this work help cure patients.

6.5 PhD Experience

The PhD experience while often times extremely frustrating and challenging, has in the end been a truly rewarding experience. I came to graduate school to further my scientific knowledge and skills to make myself more marketable in the job market. I believe my passion for science went beyond the undergraduate level. As an undergraduate in the academic and industry settings I was comfortable reading and for the most part understanding scientific literature. However, journal clubs became difficult when thought provoking questions were asked that required more critical thinking of each experiment and

detail . I think the biggest leap and change I have seen through my graduate studies, is that my mentor, faculty members, and peers have really challenged and helped me excel at critical thinking. The ability to evaluate, ask challenging questions, and critique my work and the work of others is a tremendous skill I have gained in graduate school. I think the growth in looking at a scientific protocol and being able to distinguish why each step is performed is another tremendous skill graduate school has taught me. I also leave graduate school much more confident in scientific presentations whether it be seminars or poster sessions. Overall, there is not much I would change about my graduate experience. I do think if I could go back I would look to establish more collaborations during my project to expose myself to even more scientific techniques and gain knowledge from experts in their field. I would also start my *in vivo* work much earlier in the graduate process as I many times at poster sessions or seminars I was told that was the one area missing from my data that held me back from winning awards at conferences. In the end I am truly grateful for the experiences I have had at IUSM that have shaped the scientist I have become and believe the skills I have learned along the way will allow me to succeed in future endeavors.

APPENDIX. PERMISSIONS



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CURRICULUM VITAE

Robert Craig Peery

EDUCATION

August 2014 – January 2020 Indiana University
PhD. in Pharmacology
Minor: Cancer Biology

August 2009 - May 2014 Purdue University
B.S. in Biology

RESEARCH EXPERIENCE

May 2015 – Present PHD Candidate in Dr. JT Zhang's Lab

-Performed optimization of two different survivin inhibitor scaffolds

-Investigated the relationship between docetaxel resistance and the inhibitor of apoptosis family member survivin in the context of prostate cancer

-Co-authored manuscript published in *Cancer Research* detailing the direct survivin inhibitor discovered in Dr. JT Zhang's Lab

-Published manuscript in *Drug Discovery Today* detailing the past therapeutic targeting of survivin and its future promise as a cancer drug target

-Awarded Cancer Biology Training Fellowship and IUSM Graduate Travel Award

-Completed requirements for Cancer Biology Minor

March 2015 – May 2015 Lab Rotation in Dr. Tao Lu's Lab

-Utilized Validation-Based Insertional Mutagenesis (VBIM) technique to discover drug resistance genes in breast cancer cells

-Studied the role of phosphorylation of methyltransferase PRMT5 in colon cancer cells

Jan. 2015 – March 2015 Lab Rotation in Dr. John Turchi's Lab

-Determined the effect of Rad51 inhibitors on lung cancer cells viability and Rad51 DNA binding activity

-Performed spontaneous chalk talks to update several labs members on my research throughout rotation

-Wrote short manuscript for my PI to summarize findings from my rotation

Oct. 2014 – Dec. 2014 Lab Rotation in Dr. JT Zhang's Lab

-Developed an assay to assess the native quaternary structure of 14-3-3σ by PFO-gel electrophoresis

-Determined candidates of small molecule inhibitors with the ability to disrupt the dimerization of 14-3-3σ *in vitro*

PUBLICATIONS

Qi, J., Dong, Z., Liu, J., **Peery, R. C**., Zhang, S., Liu, J.-Y., & Zhang, J.-T. Effective Targeting of the Survivin Dimerization Interface with Small-Molecule Inhibitors. *Cancer Research, 76*(2):453-462, 2016 Jan.

Peery, R. C., Liu, J., & Zhang, J.-T. Targeting survivin for therapeutic discovery: the past, the present, and the future promises. *Drug Discovery Today* (2017), doi:10.1016/j.drudis.2017.05.009

Peery, R. C., Dai, M., & Zhang, J.-T. Optimization of LQZ-7 Survivin Dimerization Small-Molecule Inhibitor Backbone. *In preparation.*

Peery, R. C., Dai, M., & Zhang, J.-T. Structure Activity Relationship Survivin Inhibitor LQZ-7F Backbone. *In preparation*.

ABSTRACTS & POSTER PRESENTATIONS

Peery, R.C. & Zhang, J.-T. (2017, May). *Targeting Survivin to Overcome Docetaxel Resistance in Prostate Cancer.* Poster Presentation at IU Cancer Research Day. Indianapolis, Indiana.

Peery, R.C. & Zhang, J.-T. (2018, April). *Targeting Survivin to Overcome Docetaxel Resistance in Prostate Cancer.* Poster Presentation at American Association of Cancer Research (AACR) Annual Meeting. Chicago, Illinois**.**

Peery, R.C. & Zhang, J.-T. (2018, May). *Targeting Survivin to Overcome Docetaxel Resistance in Prostate Cancer.* Poster Presentation at IU Cancer Research Day. Indianapolis, Indiana.

Peery, R.C. & Zhang, J.-T. (2018, May). *Targeting Survivin to Overcome Docetaxel Resistance in Prostate Cancer.* Poster Presentation at Great Lakes Drug Metabolism & Disposition Group. Indianapolis, Indiana**.**

Peery, R.C. & Zhang, J.-T. (2018, October). *Targeting Survivin to Overcome Docetaxel Resistance in Prostate Cancer.* Poster Presentation at Indiana Basic Urological Research annual meeting. West Lafayette, Indiana.

SYMPOSIA

Peery, R.C. & Zhang, J.-T. (2018, October). *Targeting Survivin to Overcome Docetaxel Resistance in Prostate Cancer.* Selected Podium Symposium Presentation at Indiana Basic Urological Research annual meeting. West Lafayette, Indiana.

AWARDS

Cancer Biology Training Program Fellowship. Awarded by IU Simon Cancer Center. $28,000. August 2017- August 2018.

IUSM Graduate Student Travel Award. Awarded by IUSM Graduate Division for Travel to AACR 2018 Meeting. $500. 2018.